

Human Pancreatic Colipase: Structural, Evolutionary and Immunological Relationships to Other Colipases

Berit Sternby



Lund 1984



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HUMAN PANCREATIC COLIPASE:
Structural, evolutionary and immunological
relationships to other colipases

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Abstract Purification and characterization of human pancreatic (pro)colipase are described. Isolation of human procolipase was made possible by immunoaffinity chromatography. Amino acid sequences of the human colipase ₈₆ , the human propentapeptide and the N-terminal part of dogfish procolipase are presented. The sequence homology between human (pro)colipase and other (pro)colipases (porcine, equine, dogfish, chicken) is pronounced. According to four proposed criteria evidence for colipase was found in all primitive vertebrates examined: Greenland shark, rayfish, ratfish and the hagfish; the latter is the most primitive vertebrate. Colipase is thus a phylogenetically old protein and appears simultaneously with the bile salts/alcohols in the hagfish. The criteria for the existence of colipase were also fulfilled by an extract of guinea pig pancreas, which species is reported to lack classical pancreatic lipase. Colipase/lipase interactions were proved not to be species specific, in contrast to an earlier report. Radioimmunoassays for human pancreatic colipase, lipase and phospholipase A ₂ were developed, used for determination of their concentrations in normal human plasma and urine and for characterization of their molecular forms in plasma.		
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To

Malin, Jakob, Johan

Att erkänna sin okunnighet
är ett av de vackraste och säkraste
bevis på gott omdöme, som jag känner

Michel Eyquem de Montaigne

1533-1592

CONTENTS

LIST OF PAPERS	5
A. INTRODUCTION - GASTROINTESTINAL LIPID DIGESTION	7
Pancreatic lipase	9
Pancreatic phospholipase A ₂	9
Pancreatic carboxyl ester lipase	10
Pancreatic colipase	10
B. PRESENT INVESTIGATIONS	11
Purification of human colipase	12
Primary sequence of human colipase	13
Isolation of human procolipase by immunoaffinity chromatography	17
Purification of colipase from dogfish	17
Evolutionary studies on colipase	18
Comparative studies of colipase/lipase	20
Radioimmunoassays for human colipase, lipase and phospholipase A ₂	21
GENERAL SUMMARY	24
ACKNOWLEDGMENTS	26
REFERENCES	28
APPENDIX: PAPERS I-VII.....	35



LIST OF PAPERS

This thesis is based on the following papers referred to by their Roman numerals in the text.

- I. Purification and characterization of human pancreatic colipase.

Berit Sternby and Bengt Borgström.

Biochim. Biophys. Acta 572 (1979) 235-243

- II. The primary sequence of human pancreatic colipase.

Berit Sternby, Åke Engström, Ulf Hellman, Anatholy Vihert, Nils-H. Sternby and Bengt Borgström.

Biochim. Biophys. Acta 784 (1984) 75-80.

- III. One step purification of procolipase from human pancreatic juice by immobilized antibodies against human colipase⁸⁶.

Berit Sternby and Bengt Borgström

Biochim. Biophys. Acta (1984). In press.

- IV. Purification and characterization of pancreatic colipase from the dogfish (*Squalus acanthius*).

Berit Sternby, Åke Engström and Ulf Hellman

Biochim. Biophys. Acta (1984). Submitted.

- V. Evolutionary studies on pancreatic colipase.

Berit Sternby, Anita Larsson and Bengt Borgström

Biochim. Biophys. Acta 750 (1983) 340-345

- VI. Comparative studies on the ability of pancreatic colipases to restore activity of lipases from different species.

Berit Sternby and Bengt Borgström

Comp. Biochem. Physiol. 68B (1981) 15-18

VII. Immunoreactive pancreatic colipase, lipase and phospholipase A₂ in human plasma and urine from healthy individuals.

Berit Sternby and Bo Åkerström

Biochim. Biophys. Acta (1984). Submitted.

These papers can be found from page 35 to 111. Those of the papers which have already been published have been reproduced with kind permission from Elsevier/North Holland Biomedical Press, The Netherlands and Pergamon Press Ltd, Great Britain.

INTRODUCTION - GASTROINTESTINAL LIPID DIGESTION

Digestion of lipids starts in the stomach, where lingual lipase (1) is responsible for the enzymatic activity. The lingual lipase originates from the lingual serous glands in the back of the tongue. The earlier reported gastric lipase activity is probably identical with that of lingual lipase. The lingual lipase prepares dietary fat for more efficient digestion in the small intestine, changing its surface properties.

The product of this hydrolysis (about 30% of the original triglycerides, now converted to diglycerides and fatty acids) reaches the intestine as a lipid emulsion consisting of a mixture of triacylglycerols, partial glycerides, fatty acids and phospholipids. The amphiphilic phospholipids are gathered on the surface of the lipid emulsion, and provide some stability to the lipid-water interface. In the duodenum the lipid particles are mixed with bile and pancreatic juice, the latter containing the enzymes responsible for continued hydrolysis.

The mammalian exocrine pancreas synthesizes, stores and secretes approximately twenty proteins which take part in intestinal digestion. Human pancreatic juice (approx. 1 litre/day) contains about 20 g protein, most of which is enzymatic protein. Of all organs the pancreas has the highest capacity per weight to synthesize proteins (2). Among these digestive proteins are three enzymes and one cofactor all concerned with lipolysis. Many pancreatic proteins are produced and secreted as zymogens, which are activated in the duodenum. The activation process is started by enterokinase, which converts trypsinogen to trypsin. Trypsin is the main activator for the other zymogens.

The lipolytic pancreatic proteins are lipase (EC 3.1.1.3), phospholipase A₂ (EC 3.1.1.4), carboxyl ester lipase (EC 3.1.1.13), and colipase. Phospholipase A₂ and colipase are secreted in proforms (3,4). Intestinal lipid digestion is a continued hydrolysis of triglycerides and diglycerides to a mixture consisting mainly of monoglycerides and fatty acids. The products are dispersed in liposomes and micelles in the presence of bile and are subsequently absorbed. These processes enable a vast array of other fat-soluble substances to be efficiently absorbed. Among these are the fat-soluble vitamins and other nonpolar lipids present in minor quantities.

Bile salts are not necessary for the activity of lingual lipase. Pancreatic lipase has no lipolytic ability in the presence of bile. The lipase activity, however, is regained in the presence of colipase. The actions of the bile salts appear to be a cleansing of the lipid-water interface of denaturated proteins and solubilization of the products of lipid hydrolysis. Bile salts also stabilize lipase by preventing it from unfolding at the lipid/water interface.

There are indications that the above lipolytic system is phylogenetically old. The hagfish, the most primitive species among vertebrates, has no well defined pancreatic gland, but has exocrine cells of pancreatic type spread throughout its gut wall (5) and Brockerhoff (6) showed the digestive lipase of lobster, in a purified form, will not hydrolyse tributyrin unless a protein cofactor is present. Bovine albumin can replace this cofactor.

All vertebrates have bile salts or alcohols. The bile salts in the hagfish are the most primitive ones (7). Below birds, all vertebrates (so far investigated) have a gall bladder, but several mammals, e.g. rat and elephant lack this organ.

Thus, there are anatomical differences in the gastrointestinal tract among the vertebrates. The process of lipid digestion, however, seems to be very similar, although the amount and composition of pancreatic enzymes, bile salts and dietary lipids differ. After the lipid digestion has occurred, the products are absorbed. The lipolytic products (fatty acids and monoglycerides) are taken up in the epithelial cells in the small intestine of the vertebrate organism. Several comprehensive reviews of lipid digestion and absorption have recently been published (8,9).

Pancreatic lipase

Pancreatic lipase is responsible for most of the lipolytic capacity in the adult mammalian pancreas hydrolyzing 1,3 ester bonds of di- and triacylglycerols. Digestive enzymes similar to pancreatic lipase can be found in evolutionarily ancient organisms like the marine clam (10). On the basis of specificity studies, a lipase of pancreatic type appears to be the major triglyceride digesting enzyme in insects, crustaceans and many fish species. One fish, the leopard shark (11), is reported to lack pancreatic lipase, and only have a bile-salt dependent lipase as its lipid digesting enzyme. This would probably be identical to the carboxyl ester lipase of other species. Among mammals the guinea pig is reported to have two pancreatic lipases which also have phospholipase activity, the proportion of the enzyme activities being 1:1 (12). Pancreatic lipase (for a recent review see (13)), is inactive in the presence of bile salts and/or phospholipids, the main components of bile.

Pancreatic phospholipase A₂

Pancreatic phospholipase A₂ hydrolyzes the fatty acid at the 2-position of dietary and biliary phospholipids. Studies of its chemistry and interactions in model systems are far advanced

compared to other lipolytic enzymes. However, the knowledge of the biology and ecology of pancreatic phospholipase A₂ as it operates in the intestine is very fragmentary.

Pancreatic phospholipase A₂ is secreted in a proform first observed by Rimón and Shapiro (3), who could not find enzyme activity in fresh ox pancreas. Among mammals guinea pig pancreas is reported as a species lacking classical secretory phospholipase A₂ (14). Most of the work on pancreatic phospholipase A₂ has been carried out by de Haas and coworkers (15).

Pancreatic carboxyl ester lipase

Carboxyl ester lipase was first found in rat pancreatic juice (16). Together with lipase, colipase and phospholipase A₂, carboxyl ester lipase plays a role in lipid digestion. The functional importance of carboxyl ester lipase is probably related to its broad substrate specificity (17,18). This enzyme is probably identical to non-specific lipase, cholesterol esterase and bile salt-dependent lipase and has an absolute need for trihydroxy bile salts for activity.

Rat pancreatic carboxyl ester lipase is said to have a function in synthesis of cholesterol esters, perhaps of importance for their uptake in the enterocyte (19). Bile salt-stimulated lipase from human milk (20) may be identical with human pancreatic carboxyl ester lipase. The milk enzyme is especially important in the perinatal period, when the pancreatic function is low.

Pancreatic colipase

Colipase, a low-molecular weight, heat- and acid-stable protein, has been subject to much research during the last fifteen years. (For a recent review, see (21).) Already in 1910, Rosenheim (22) discovered that after filtration, a glycerol extract of porcine pancreas lost its lipolytic activity against a

neutralized (i.e. soap stabilized) olive oil emulsion. This activity was restored after recombination of the sediment and the filtrate. The filtrate was found to contain a dialysable and thermostable coenzyme, the activity of which was lost upon incineration.

These findings by Rosenheim were forgotten and the coenzyme for lipase, was not rediscovered until 1963 by Baskys et al. (23) and subsequently named colipase by Maylie et al. (24). In the late sixties and early seventies interest was focused on colipase in several laboratories (25-27) and colipase has been identified or purified from many species (21).

Colipase has been shown to be present in a proform, procolipase, in pancreatic juice or gland of several species among higher vertebrates. The procolipase is converted to an active form by tryptic digestion. This activation of the proform of colipase results in a cofactor, which is required so that pancreatic lipase is able to be enzymatically active under in vivo conditions.

This thesis deals with the purification and characterization of human pancreatic (pro)colipase, including its primary sequence. Studies of the interaction of different colipases with heterologous pancreatic lipases and of the evolution of colipase are also included. Radioimmunoassay methods are described which allow measurement and characterization of colipase, lipase and phospholipase A₂ in human plasma and urine.

PRESENT INVESTIGATIONS

Of pancreatic lipolytic proteins those most studied are of porcine origin. To understand fully lipid digestion in man, it is important to have available the human lipolytic proteins in high

purity. This investigation is mainly concerned with colipase from human pancreas but also pure human pancreatic lipase (paper VI) and phospholipase A₂ (28) are dealt with. Pure lipase served as 1) the lipase source for determination of colipase in all species investigated, 2) the standard for determination of lipase in plasma and urine as well as 3) the antigen used to obtain anti-human pancreatic lipase antibodies. Pure human phospholipase A₂ was used in a similar way as lipase under 2) and 3) (see above). For evolutionary studies it is of great importance to establish the molecular properties of the proteins in the animal series.

Purification of human colipase (Paper I)

Human pancreatic glands were obtained by collaboration with Professor Vihert, Moscow. These glands had been removed within 2-3h and at most 6h after death and kept frozen until use.

Colipase was purified from defatted extracts of human pancreatic glands. The purification procedure includes centrifugation, ion exchange chromatographies, gel filtration, dialysis and lyophilization. The colipase had a molecular weight of about 10,000 and an N-terminal glycine. The specific activity was about 25,000 units/mg at pH 7.0, room temperature and in the presence of a surplus of pancreatic lipase with tributyrin as substrate. Two different colipases were isolated with different pI and the most basic one (pI=6.1) was further analysed. Later work indicates, however, that the most basic colipase is a form of human colipase with 87 or more residues. The colipase₈₆ whose sequence is now determined (paper II), would be the less basic one (pI=5.8). At that time we also reported that human colipase contained a minor proportion of carbohydrate, but these observations could not be verified during the work with the sequence.

Sequence determination of human colipase₈₆ (paper II)

Human colipase₈₆ was obtained as described in paper I. The pure protein was reduced and carboxymethylated with ^3H -2-iodoacetate. This CM-colipase was cleaved using CNBr, because it was known from the amino acid composition that human colipase contains two methionines. The three peptides thus obtained were purified from the crude mixture by gel chromatography on a Sephadex G-75 column. Each fraction was checked for the amount of ^3H (cpm) and protein (absorbance at 280 nm).

Four peaks were obtained from the column. The first one, which contained uncleaved colipase, was discarded. The other three were designated CNBr I, II and III, respectively. Amino acid compositions of these three pools showed that CNBr I had no homoserine and it was shown to be the C-terminal peptide. Pool III had an amino acid composition which agreed with the amino acid sequence Gly₆-Met₁₈ of the N-terminal sequence determined in paper I. This peptide was not further analysed. The middle-sized peptide CNBr II was thus considered to be the intermediary fragment. In numbering the residues in human colipase₈₆ the N-terminal glycine was chosen as number six in order to make comparison with the equine and porcine procolipases, which have a pentapeptide in front of this glycine.

The sequencing of the whole reduced and carboxymethylated protein was successful up to residue 86, which meant 81 steps. Sequencing of CNBr II and I confirmed this result and added 5 more residues in the C-terminal part. The homology between human, porcine and equine colipases is very striking, especially from residues 43 to 87, where in all four proteins there are totally 18 substitutions. The similarity between these colipases was earlier indicated in evolutionary and comparative studies (paper V and VI). Table I shows the amino acid sequences of all colipases sequenced hitherto.

1	5	10	15	20	25	30	35	40	45	50
Man	AlaProGlyProArgGlyIle*	IleIleAanLeuGluArgGlyGluLeu*	CysMetAanSerAlaGlnCysIysSer	AanCysCysGlnHisSerSer	ArgAlaLeuGlyLeuAla*	ArgCysThrSerMetAlaSerGluAanSerGluCysSer				
Pig	ValProAspProArgGlyIle	IleIleAanLeuAanGlyGlyGluLeu	CysLeuAanSerAlaGlnCysIysSer	AanCysCysGlnHisAspThrIleLeuSerLeuLeu	ArgCysAlaIleuIysAlaArgGluAanSerGluCysSer					
Horse A	ValProAspProArgGlyVal	IleIleAanLeuGluAlaGlyGluIle	CysLeuAanSerAlaGlnCysIysSer	GluCysCysHisGlnGluGluSerLeuSerLeuAla	ArgCysAlaAlaIysAlaArgGluAanSerGluCysSer					
Horse B	ValProAspProArgGlyVal	IleIleAanLeuGluAlaGlyGluIle	CysMetAanSerAlaGlnCysIysSer	GluCysCysHisGlnGluGluSerLeuSerLeuAla	ArgCysAlaAlaIysAlaArgGluAanSerGluCysSer					
Dogfish	AlaProGlu - ArgGlyLeu	PheLeuAanLeuSerAlaGlyGluLeu	CysValGlySerPheGlnCysIysSer	SerCysCysGlnHisGlnThrGlyLeuSerLeuAla	ArgCysAlaAlaIysAlaArgGluAanSerGluCysSer					
Chicken		GlyLeu	IlePheAanLeuAanThrGlyGluLeu	x LeuGlnSerAlaGlnCysIysSer	GluAspSerGlyLeuSerLeuAla	x Cys				
51	55	60	65	70	75	80	85	90	95	
Man	ValLysThrLeuTyrGlyIle*	TyrTyrIysCysProCysGluArgGlyLeuThrCysGluGly*	AspIysThrIle*	ValGlySerIleThrAanThrAanPheGlyIleCysHisAspAla*	Gly*					
Pig	AlaPheThrLeuTyrGlyVal	TyrTyrIysCysProCysGluArgGlyLeuThrCysGluGly	AspIysSerLeu	ValGlySerIleThrAanThrAanPheGlyIleCysHisAsnVal	Gly	ArgSerLysSer				
Horse A	AlaTrpThrLeuTyrGlyVal	TyrTyrIysCysProCysGluArgGlyLeuThrCysGluVal	AspIysThrLeu	ValGlySerIleMetAanThrAanPheGlyIleCysPheAspAla	Ala	ArgSerGluGlxArg				
Horse B	AlaTrpThrLeuTyrGlyVal	TyrTyrIysCysProCysGluArgGlyLeuThrCysGluVal	AspIysThrLeu	ValGlySerIleMetAanThrAanPheGlyIleCysPheAspAla	Ala	ArgSerGluGlxArg				

TABLE I

Amino-acid sequences of human procolipase (paper II, III), porcine procolipase (paper II; 29, 31), equine procolipase A and B (31, 51), dogfish procolipase (paper IV) and chicken colipase (52). The sequences are arranged to obtain maximal homology. (-) = residue which is deleted and (x) = residues which are not defined. Residues which differ from the most frequent one in the corresponding position are underlined. Conserved substitutions are visualised with * on the residue.

The amino acid sequence of human pancreatic colipase showed the presence of 86 residues. This is in accordance with that of the equine isocolipases. These facts indicated that two amino acid residues had been overlooked in porcine colipase, which in its first sequence determination (29) contained 84 residues. Redetermination of the sequence of porcine procolipase gave a leucine in position 37 and a serine in position 50. In a staphylococcal proteinase fragment, 49-64, of porcine colipase, de Caro et al. (30) also found a serine in position 50. This result does change the numbering of porcine colipase residues which means that porcine colipase₈₄ has 86 residues. For example, several papers (21) have appeared, where His₈₆ in porcine colipase is involved. This residue should now be His₈₈. In horse colipases this residue is a phenylalanine which might change the interpretation of the importance of this residue. Other residues, which are well investigated are the three tyrosines, which have the numbers 55, 58 and 59. These residues are proposed by many authors to be involved in the interface recognition site (21). They are all conserved in the four colipases, whose complete sequence are known.

A residue in human colipase, which differs from the porcine and equine colipases is residue 52, a lysine in human colipase, but phenylalanine in porcine and tryptophan in equine colipases. These aromatic residues were found to be of critical importance for the binding of colipase to lipid-water interphases (31). Local charge differences contribute to small differences obtained e.g. by heterologous colipase/lipase system (paper VI) or in colipase/heterologous antibody interactions (paper VII).

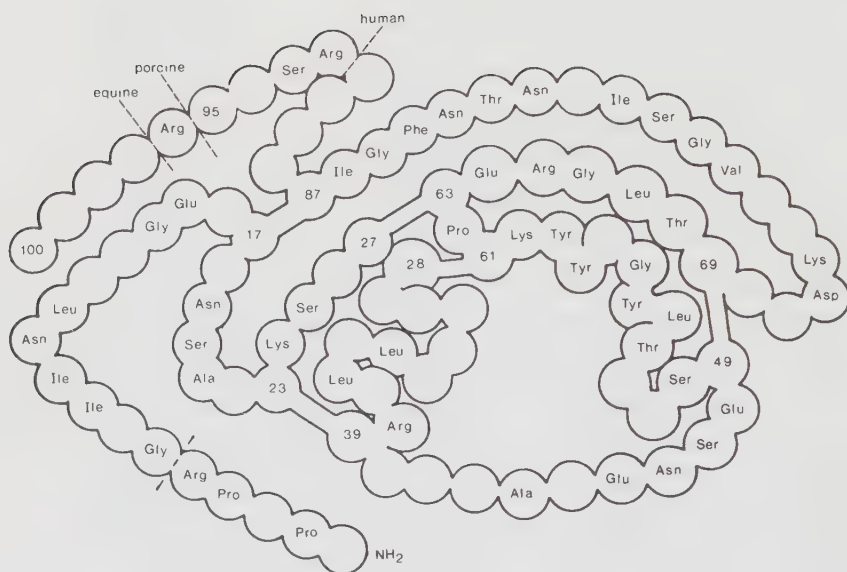


Figure 1. Bidimensional representation of a hypothetical folding of the colipase. Amino acid residues in common for human, porcine and equine A and B procolipases are inserted. The last C-terminal amino-acid established in each of the procolipase sequences is marked (-----).

So far, equine and porcine colipases have been crystallized. With these results and the primary sequence of human colipase₈₆ it is possible to make a data plot for a possible three-dimensional structure of human colipase. The three-dimensional structure visualizes how an amino acid substitutions change the conformation of one colipase compared to another. A hypothetical bidimensional folding of colipase is shown in Figure 1. Residues in common for the four proteins, whose whole sequences are known are inserted. The original bidimensional picture was worked out for equine and porcine colipase (31).

Isolation of human procolipase from pancreatic juice by immunoaffinity chromatography (paper III)

Several attempts were made to isolate human procolipase (Sternby, unpublished). It was not successful until a sufficient amount of human colipase was obtained to prepare an immunoaffinity column. Human colipase₈₆ was coupled to CNBr-activated Sepharose 4B (32). This affinity column was used to purify antihuman colipase₈₆ antibodies from rabbit antiserum. The pure antibodies were then coupled to CNBr-activated Sepharose 4B (see above) and the immunoaffinity column thus obtained was used to isolate human procolipase.

The proform of human colipase has a different N-terminal sequence Ala-Pro-Gly-Pro-Arg- compared to horse and pig procolipases, whose N-terminal peptide is Val-Pro-Asp-Pro-Arg. The human pentapeptide has a net single positive charge but the other propeptides isolated so far are neutral. Human pancreatic phospholipase A₂ also has a proform (33), the N-terminal sequence of which differs somewhat from that of the zymogens from other species. Whether these differences are of any physiological significance has not as yet been established.

Attempt by Rathelot et al. (34) to isolate human procolipase by immunoaffinity chromatography (with antiporcine procolipase antibodies) from lyophilized human pancreatic juice were unsuccessful and gave two forms of colipase with N-terminal glycine. Possibly, in their experiments the juice was left too long before analysis thus allowing proteolytic degradation.

Purification and characterization of dogfish colipase (paper IV)

Extracts from dogfish (*Squalus acanthias*) pancreas were, in the preliminary steps, purified according to a procedure used for the preparation of insulin from hagfish (35). From each purifica-

tion step the fractions were analysed for colipase activity and these fractions were pooled, and further purified as described. The pure dogfish colipase had a high isoelectric point 10.2, which is 2-3 pH units higher than for other colipases (paper VI). The molecular weight was calculated to 9108-9383. The N-terminal sequence was determined to be Ala-Pro-Glu-Arg-. This indicates that colipase from dogfish is also secreted in a proform, the activation peptide, however, being a tetrapeptide.

So far, procolipase has been obtained from all higher vertebrates investigated except chicken. Two types of proforms of colipase have been isolated having an N-terminal Val-Pro-Asp-Pro-Arg (pig and horse) or Ala-Pro-Gly-Pro-Arg (man). The dogfish data indicate that the proform of colipase exists in species lower than mammals. The proform of colipase thus seems to be phylogenetically old. Recently Guy et al. (36) traced activation peptides to pancreatic endoproteases in the dogfish. Also in pancreas from ratfish (*Chimaera monstrosa*) Nilsson and Fänge (37) found pancreatic proteases (e.g. trypsin-like enzymes) after activation by bovine trypsin. The partial amino acid sequence of dogfish colipase shows great homology with other colipases sequenced (four mammalian and one avian). The colipase from dogfish pancreas is, so far, the only characterized fish colipase. A great homology among the colipases is seen (Table I).

Evolutionary studies on pancreatic colipase (paper V)

Patton et al. (38) showed that dogfish pancreas contained colipase activity. Colipase activity was also found in trout by Leger et al. (39). Colipase activity has not reported in any lower vertebrates than fish. Another finding by Patton et al. (11) was that in leopard shark there is no pancreatic lipase, just a bile salt dependent triacylglycerol lipase. These findings raise the possibility that lower vertebrates and invertebrates have no colipase/lipase system, but simply a bile salt-dependent

lipase. Among mammals the guinea-pig is reported as a species lacking both classical pancreatic lipase (14) and phospholipase A₂ (14). This raises the question: does it have a pancreatic colipase? In an extract of guinea pig pancreas a colipase activity was found. Gel filtration of this extract on Sephadex G-50 gave a peak with colipase activity, which corresponded to a M_w about 10,000, like other colipases and was not correlated with high protein concentration. This peak with colipase activity was further analysed with the radioimmunoassay method described for human pancreatic colipase (paper VII) and a symmetrical peak was obtained, which fitted well with the pattern obtained by measuring the colipase activity (Sternby, unpublished).

Four criteria for the presence of colipase were proposed and have been used to check if colipase could be found in pancreatic glands from dogfish, Greenland shark, rayfish, ratfish, intestine and intestinal content from hagfish and gastric content from crayfish. The criteria were 1) Colipase restores the activity of human and porcine lipases inhibited by bile salt. 2) Colipase cross-reacts with antisera to human and porcine colipases. 3) The restoration of lipase activity, inhibited by bile salt, in the tributyrin assay system, is prevented by antiserum to colipase. 4) colipase is a heat-stable, low-molecular-weight protein (molecular weight about 10000 by gel filtration). All four criteria were fulfilled by all species except crayfish. Colipase thus appears in all vertebrates studied, including the hagfish, which has no well organized pancreas. Colipase was not found in crayfish, but possibly there are molecules acting like colipase even in invertebrates. Brockerhoff (6) found indications for such a molecule in lobster. What may determine the need for colipase, is the type of bile detergents. Invertebrates have acylpeptides as detergents (7) whereas steroid alcohols and acids appear as detergents in the bile in vertebrates.

All these data together show that colipase is probably as old as the bile salts/alcohols and that it is a well conserved molecule. Any colipase checked thus cross-reacts with antibodies against human colipase⁸⁶ in immunological assay systems, like ELISA and RIA with PEG 6000, which are sensitive enough to detect one colipase - one antibody complex. When the antigens are of low molecular weight like colipase it is important to add polyethyleneglycol 6000 to ensure that the antigen-antibody complex precipitates (40). The strength in the binding of one colipase to antibodies against other colipases is, however, much lower (paper VII).

Comparative studies of pancreatic colipase (paper VI)

P.C. Lee stated in 1978 (41) that canine colipase does not activate pancreatic lipases from man and rat. To confirm these results pure or partially pure lipases and colipases from dog, man, pig and rat pancreatic gland or juice, were checked in all combinations. The result was that colipase from one species can restore the ability of bile salt depressed lipases from other species to hydrolyse lipids.

There was a tendency, however, that in a homogeneous system (i.e. lipase/colipase for the same species) less colipase was needed to get the maximal lipase activity. When the proteins come from the same species, a broader pH optimum was obtained with a lower ratio of colipase to lipase. The different results obtained by Lee and us may be explained as follows. The experiments by Lee were undertaken at pH 8.8, at which pH the activity of the colipase-lipase system is low and the stimulation by colipase is rather unimportant and due to a weak binding of colipase (38). Lee also found that his canine colipase inhibited lipase activity at concentrations ten times that used by us. This inhibition

disappeared when the canine colipase solution was diluted, which raises some doubts about the purity of his canine colipase preparation.

Radioimmunoassays of human colipase₈₆, lipase and phospholipase A₂ (paper VII)

Radioimmunoassays for each of the three lipolytic proteins from human pancreas are described. Polyethyleneglycol 6000 could be used in the assay, since all three proteins are antigens of relatively low molecular weight. For these radioimmunoassays, pure human colipase₈₆, lipase and phospholipase A₂ were used, first to raise the respective antibodies in rabbits, and then for purification of the resulting antibodies by affinity chromatography on CNBr-activated Sepharose 4B. The radioimmunoassays were checked both with purified antibodies and with whole antiserum. The same results were obtained, whether the antibodies had been purified or not.

It has long been known that human plasma and urine contain amylolytic activity, which partly comes from the pancreatic gland (42). This indicates a leakage of exocrine pancreatic proteins to the circulation. Normally very small amounts of pancreatic proteins are detected in plasma and still less in urine, but in acute pancreatitis high levels are obtained (43,44). Increased levels of colipase, lipase and phospholipase A₂ in acute pancreatitis (45-47) and low values of pancreatic lipase in plasma from insulin-dependent diabetics (48), have also been reported but most of the methods used were not specific. The radioimmunoassays in paper VII were used to determine the amount of each of the three proteins in plasma and urine from healthy individuals. The amounts obtained for each of the three proteins were low, which agree well with the fact that pancreas does only leak small amounts of exocrine proteins to the blood under non-pathological

circumstances. It also shows that only small amounts of the proteins appear in the urine, indicating a high level of tubular reabsorption.

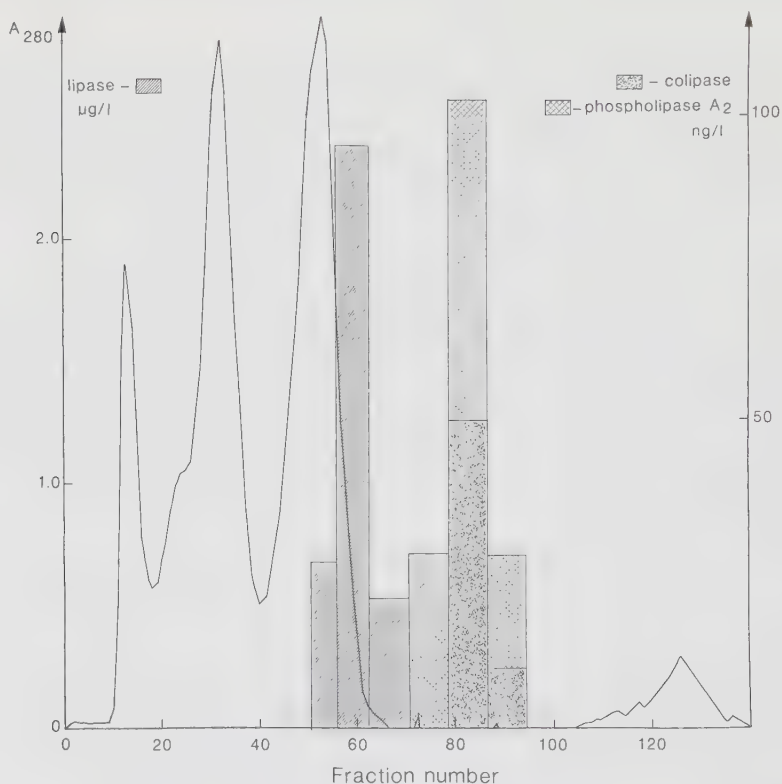


Fig. 2. Gel filtration of normal human plasma on a Sephadex G-200 column. Fractions were pooled and concentrated and the amounts of colipase, lipase and phospholipase A₂ were determined by radioimmunoassays.

For characterization of the three lipolytic proteins in human plasma, the following experiments were carried out. Normal human plasma was applied to a Sephadex G-200 column. The fractions were pooled and concentrated. The radioimmunoassays were used to

analyse the eluate of the gel chromatography, and thus the size distribution of colipase, lipase and phospholipase A₂ in human plasma. All three seem to appear in a monomer form. No aggregation or non-specific binding to macromolecular complexes was seen (see Fig. 2). Furthermore, plasma without any additives was centrifuged as above and the distribution between lipid-depleted and lipid-enriched portion was checked with each of the radioimmunoassays. None of the proteins could be found in higher amount in the lipid-enriched portion. ¹²⁵I labelled antigens were each incubated for 12 h at room temperature with plasma. The equilibrated plasma was then centrifuged and divided in a lipid-enriched and a lipid-depleted portion, and the partition of respective antigen was analysed. No indications were found for an uneven distribution.

In in vitro studies with an egg phosphatidylcholine monolayer as lipase substrate a much weaker binding between the lipids and the proforms of colipase and phospholipase A₂ compared to the activated proteins (49) was obtained. These results are in accordance with what is found for the pancreatic proteases which occur in plasma as proforms.

Leger et al (50) reported, however, that immunoreactive colipase in porcine plasma is bound to a protein or protein complex, but their proof for this result is weak. One ml of porcine plasma was used for gel filtration, and in one single fraction (1.3 ml) from this column was found a colipase concentration of 1 ng/ml (detection limit 0.5 ng/ml), although they reported that the average concentration of colipase in pig plasma was as high as 28.6 ng/ml,

The radioimmunoassays were also used to check cross-reactivity between the anti-human protein anti-bodies and the corresponding porcine proteins. As expected, these heterologous interactions

were weaker than the homologous interactions. However, they were specific enough to distinguish between porcine procolipase and colipase₈₇ as well as between porcine prophospholipase A₂ and phospholipase A₂.

GENERAL SUMMARY

Purification and characterization of human pancreatic colipase₈₆ and procolipase are described. The purification of the procolipase was made possible by the development of a one-step purification method, using an immunoaffinity column.

The amino acid sequence of human colipase₈₆ was determined as well as the additional five N-terminal amino acid residues found in the proform of human colipase. The human N-terminal propentapeptide differs from the neutrally charged porcine and equine propeptides by two amino acid substitutions and by a single positive charge. Except for this discrepancy, the sequence homology between human, porcine and equine procolipases is pronounced.

Dogfish colipase was also isolated and its 39 N-terminal amino acid residues were determined. A proform of this colipase was found with a neutral tetrapeptide as its N-terminal propeptide. Thus during evolution (fish to mammals) an additional proline residue has been inserted on the N-terminal side of the arginine₅-glycine₆ bond, the N-terminal bond which is cleaved upon activation of the procolipase.

For the study of colipase in primitive vertebrates specific criteria for the existence of colipase were proposed. According to these criteria indications were found for the presence of colipase in the following primitive vertebrates: Greenland shark, rayfish, ratfish and hagfish; the latter is the most primitive of

all vertebrates. Thus, colipase is a phylogenetically old protein, which appears simultaneously with the bile acids/alcohols. Using the same criteria, colipase was also found in guinea pig, a species which is reported to lack a classical pancreatic lipase.

The ability of colipase to restore bile-salt depressed pancreatic lipase activity was found not to be species specific. This is in contrast to an earlier report.

Radioimmunoassays for human colipase, lipase and phospholipase A₂ have been developed, and used to determine the concentrations of these pancreatic proteins in normal human plasma and urine. These assays allowed characterization of the molecular forms of these proteins in normal human plasma.

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PURIFICATION AND CHARACTERIZATION OF HUMAN PANCREATIC COLIPASE

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Summary

Two colipases, named colipase I and colipase II, have been isolated from extracts of human pancreatic gland. The two proteins can be separated by ion-exchange chromatography, isoelectric focusing and slab technique gel electrophoresis. The result of this study indicates that the two colipases, both of which are glycoproteins, have identical amino acid compositions. The *pI* values were found to be 6.1 for colipase I and 5.8 for colipase II. The different colipases have also been found in human pancreatic juice. The N-terminal amino acid was glycine for both colipase I (gland) and colipase II (juice). Only minor differences were found between the colipases isolated from gland and juice, and colipase I from gland alone was examined in detail.

Introduction

Colipase from porcine pancreas has been isolated by Maylié et al. [1], Erlanson et al. [2] and Canioni et al. [3], from bovine pancreas by Rathelot et al. [4] and from horse pancreas by Julien et al. [5]. Kimura et al. [6] reported the partial purification from human pancreatic juice of a protein activator of human pancreatic lipase. The overall yield of the protein activator was about 0.5% with at least an 80-fold increase in specific activity. Due to the difficulties in obtaining sufficient amounts of human pancreatic juice it was decided to purify human colipase from the human pancreatic gland obtained at autopsy. Pancreatic gland contains about 0.1 mg colipase per g. The total yield was around 30% and the degree of purification was about 400 times.

Material and Methods

The raw material for colipase purification was obtained by collecting glands during post-mortem examinations, which occurred within 10 h after death.

Human pancreatic juice was obtained by courtesy of Dr. Kjell Ohlson of the Department of Surgery, Allmänna Sjukhuset, Malmö. The juice was collected over ice and lyophilized. Organic solvents were distilled before use. Sephadex G-100 and SP-Sephadex C-25 were purchased from Pharmacia, Sweden and DEAE-cellulose (microgranular DE52) from Whatman. Tributyrine was purchased from B.D.H. and used without further purification. Sodium taurodeoxycholate was synthesized in this department according to Hofmann [7]. Spectrapor I membrane was used for dialysis (Spectrum Medical Industries Inc., Los Angeles).

Assay for colipase

Colipase activity was determined as earlier described by Erlanson et al. [8], using tributyrine as substrate with automatic titration of the fatty acids released. 1 colipase unit corresponds to the liberation of 1 μ mol butyric acid per min under the conditions described. The titrator was a Mettler pH-state and the lipase source was porcine pancreatic lipase B, kindly supplied by Dr. Jakob Donnér, Lund.

Determination of protein

Protein concentration was determined by measuring the absorbance at 280 nm, using $E_{280}^{1\%} = 10.0$.

Determination of carbohydrate

Hexosamine content was analysed in a DURRUM D500 amino acid analyser after 10 h hydrolysis at 110°C in 4 M HCl. Neutral sugars were determined by a method described by Axelsson et al. [9]. The amount of *N*-acetylneuraminic acid was determined according to Jourdian et al. [10].

Amino acid analyses

The amino acid analyses were performed in a DURRUM D500 amino acid analyser. The calculations are based on 18, 24 and 72 h hydrolyses in 6 M HCl at 110°C.

Electrophoresis

Electrophoresis was carried out by the slab technique using a 15% polyacrylamide gel at pH 8.9 as described by Maurer [11]. The equipment for the electrophoresis was obtained from Pharmacia, Sweden. The gels were stained with Coomassie Brilliant Blue G250 [12].

Molecular weight determination

Rough determinations of the molecular weights were made by gel chromatography on a Sephadex G-100 column and by SDS gel electrophoresis with ovalbumin, trypsin and porcine colipase as standard proteins. Probable values for the molecular weights were calculated from the amino acid composition according to Delaage [13].

N-terminal sequence determination

The *N*-terminal amino acid sequence of colipase I from gland was determined

in a Beckman 890C sequenator. Each step was verified by both thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC). (This was kindly done by Dr. Jan-Olof Jeppson in the Department of Clinical Chemistry at Allmänna Sjukhuset, Malmö, Sweden).

Isoelectric focusing

Isoelectric focusing was performed as described by Vesterberg et al. [14]. A column of 110 ml volume from LKB-Produkter, Sweden, was used with a 1% ampholine pH gradient between pH 5 and 7 (LKB-Produkter) and sucrose as gradient stabilizer.

The ethanol circulating through the mantle was kept at 4°C in a thermostated bath. The electrofocusing was run for 48 h at 1200 V and 6 mA. The column was emptied by collecting fractions of about 1.5-ml. The pH as well as the colipase activity was checked in each fraction. The pH measurements were done at 4°C in a Radiometer (Copenhagen) pH M71 Mk2.

Results

Purification procedure

The frozen glands were left to thaw until they could be ground in a meat-mincer. After grinding three times, they were delipidated according to the method described for pig pancreatic gland [15]. The powder was dried overnight at room temperature and then stored at -20°C until used. Each preparation started with 1 kg pancreatic gland, roughly 10 glands, and gave about 100 g fat-free powder. This powder was suspended in a 50 mM citrate-HCl buffer pH 3.0 (20 ml/g powder) at room temperature and was stirred for 60 min, then centrifuged in an MSE 18 centrifuge for 30 min at 9000 rev./min and 4°C, and the pellet was discarded. The supernatant was saturated to 0.6% with (NH₄)₂SO₄ and stirred for 60 min at room-temperature, then centrifuged as described above. The supernatant was rejected, and the pellet was dissolved in 50–100 ml 10 mM sodium acetate buffer, pH 5.0.

Gel chromatography

The clear extract obtained as described above was subjected to gel chromatography on a Sephadex G-100 coarse column (K50/100) in 10 mM sodium acetate buffer (pH 5.0).

For pancreatic juice the procedure was replaced by the following treatment. After redissolving 200 ml lyophilized juice in 25 ml 50 mM sodium acetate buffer pH 5.0, containing 200 mM NaCl, 2 mM CaCl₂ and 30 mg soybean trypsin inhibitor, the solution was applied to a Sephadex G-75 column. The fraction with colipase activity from the G-100 and G-75 columns, respectively, were pooled and dialysed against 10 mM Tris-HCl buffer (pH 8.0) overnight.

Ion-exchange chromatography

The dialysed Sephadex pools were subsequently applied to a DEAE cellulose column (K 25/45 and K 15/30, respectively), equilibrated with 10 mM Tris-HCl buffer (pH 8.0) and eluted with a NaCl gradient (0–0.2 M) in the same buffer. The volumes of the gradient vessels were 2 × 1000 ml for the big column and

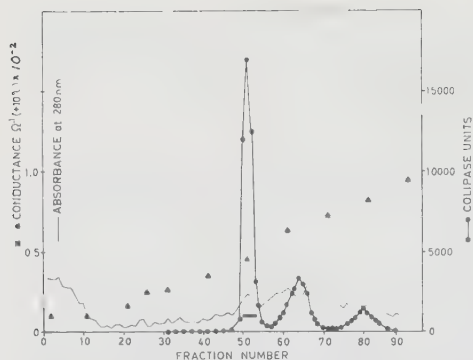


Fig. 1. Chromatography of human pancreatic gland extract (pool from Sephadex G-100) on DEAE-cellulose. The DEAE-cellulose column (2.5×45 cm) was equilibrated with 10 mM Tris-HCl buffer (pH 8.0), and eluted with a linear concentration gradient of NaCl (0.0–0.2 M). Fractions (50–53) containing pure colipase I have been marked out with a bar, Absorbance, 280 nm. ●—●, colipase units/ml, Δ , conductance Ω^{-1} (10°C).



Fig. 2. Electrophoresis on polyacrylamide slab gel of colipase I from human pancreatic gland and colipase I and II from human pancreatic juice at pH 8.9. Colipase I, gland (1 mg/ml solution, 10, 50, 25 and 5 μl , respectively; No. 1–4 from left). Colipase I and II, juice (isoelectric focusing samples; No. 5–6 from left). Colipase II, juice (pure fraction from isoelectric focusing; No. 7 from left).

TABLE I

PURIFICATION OF HUMAN COLIPASE FROM PANCREATIC GLAND

Gland (1 kg)	Total activity ^a ($\mu\text{mol/min}$)	Spec. act. ($\mu\text{mol/min per mg}$)	Protein ^b (mg)	Percent
Sup I	$2.8 \cdot 10^6$	133	21 000	100
(NH_4) ₂ SO ₄ precipitate	$2.8 \cdot 10^6$	610	4 600	100
G-100 Peak	$2.72 \cdot 10^6$	8 000	340	97
DEAE I	660 000	55 000	12	23
DEAE II	173 400	3 830	45	6
DEAE III	29 300	1 565	19	1
Colipase I after dialysis	637 000	25 000 ^c	25.5 ^d	22.8

^a Porcine lipase B used as lipase source.^b 1 mg per ml solution $\rightarrow A_{280} = 1.0$.^c Spec. act. determined after preparing an 1% solution by weight.^d Protein by weight.

2 X 250 ml for the small one. A typical elution pattern is shown in Fig. 1. The first peak with colipase activity is called colipase I and the second one colipase II and correspond, respectively, to the conductance of $4.5 \cdot 10^{-3}$ and $6.5 \cdot 10^{-3} \Omega^{-1}$ (10°C). In this example fractions 50–53 were pooled and dialysed against 20 mM NH_4HCO_3 overnight and then lyophilized. This peak was homogeneous according to slab technique gel electrophoresis (Fig. 2) and gave the pure colipase I from gland. Colipase II from gland and colipase I and II from juice were not homogeneous until after another ion-chromatographic step on an SP-Sephadex C-25 column (K 15/30) in 10 mM acetate buffer (pH 5.0) eluted with a 0–0.4 M NaCl gradient (2 X 250 ml) in the same buffer. Colipase I and II were eluted from the column at an approximate conductance of $6.0 \cdot 10^{-3}$ and $8.0 \cdot 10^{-3} \Omega^{-1}$ (10°C), respectively. The purification procedure for colipase from pancreatic gland is summarized in Table I. A third minor peak with colipase activity was seen in some chromatograms, it probably represented a degradation product and was not further analysed.

Chemical properties of colipase I and II

Amino acid composition. The amino acid composition of colipase I from the human gland is given in Table II. For comparison, the amino acid compositions for porcine colipase I and II (gland) and horse colipase (gland) are presented in the same table.

Electrophoretic behaviour. The electrophoretic mobilities of colipase I and II were examined on polyacrylamide slab gels. The two proteins were clearly separated when the electrophoresis was run at pH 8.9. Colipase I, having the lowest mobility (see Fig. 2) is more basic which is in accordance with the behaviour on the ion-exchange chromatography.

Carbohydrate contents. Analyses for hexosamine were made with the amino acid analyser and indicate the presence of at least 0.6 mol glucosamine per mol colipase. Neutral sugar determinations performed on colipase I (gland) indicated a fucose and glucose content of approx. 0.1 mol, a mannose content of at

TABLE II
AMINO ACID COMPOSITION

Amino acid	Number of residues			
	Colipase I		Horse (5)	Colipase II Pig (2) gland
	Human gland	Pig (2) gland		
Ala	5	5	10	5
Arg	2	6	5	6
Asn + Asp	11	13	12	14
1/2 Cys *	8	10	10	9
Gln + Glu	11	10	11	9
Gly	10	10	8	9
His	2	2	2	2
Ile	6	6	6	6
Leu	6	10	7	10
Lys	4	5	6	5
Met	1—2	—	2	—
Phe	1	3	2—3	2
Pro	4	3	5	3
Ser	9	11	9	10
Thr	6	6	5	5
Trp **	—	—	1	—
Tyr	3	3	3—4	3
Val	4	4	6	4
Number of residues	93—94	107	110—112	102
Total weight of residues	9 844—9 993	11 570	12 260 ± 150	11 020

* Determined as cysteic acid.

** With two different methods, no detectable amount of tryptophan was found (refs. 18,19).

least 0.3 mol and a galactose content of 0.5 mol per mol colipase. Sialic acid content determined on colipase I (gland) was found to be 0.3 mol per mol colipase.

N-terminal amino acid sequence of the colipase I from pancreatic gland. After dansylation, colipase I was sequenced to amino acid no. 24. The N-terminal sequence is presented in Table III and compared with horse colipase sequenced by Julien et al. [5] and porcine colipase II sequenced by Charles et al. [16]. The N-terminal amino acid of human colipase I (gland) is glycine and in analogy with the horse and porcine colipase this amino acid has been assigned no. 6 in Table II, although so far no human colipase with N-terminal valine has been obtained. The N-terminal amino acid for human colipase II from juice was also glycine. Amino acid no. 17 in human colipase is not cysteine but most probably aspartic or glutamic acid.

Isoelectric focusing. Isoelectric points of 6.1 and 5.8, correspond to colipase I and colipase II (gland and juice), respectively.

Ultraviolet-absorption. The ultraviolet-absorption at 280 nm of 1 mg colipase (gland or juice) in 1 ml distilled water or 6 M guanidine-HCl was 0.4, the same as for porcine colipase.

TABLE III
N-TERMINAL AMINO ACID SEQUENCE OF COLIPASE FROM DIFFERENT SOURCES
Deviations from the human colipase are underlined.

Human colipase I (this investigation)	Gly-Ile-Ile-Ile-Asn-Leu-Glu-Asn-Gly-Glu-Leu-?*	15	-Met-Asn-Ser-Ala-Gln-Cys-	23
Horse colipase [5]	Val-Pro-Asp-Pro-Arg-Gly- <u>Val-Ile-Ile-Asn-Leu-Glu-Ala</u>	15	-Gly-Glu-Ile	<u>-Cys-Met-Asn-Ser-Ala-Glu-Cys-</u>
Porcine colipase II [16]	Val-Pro-Asp-Pro-Arg-Gly-Ile-Ile-Ile-Asn-Leu- <u>Asp-Glu-Gly-Glu-Leu-Cys</u>	15	-Leu-Cys-Leu-Ser-Ala-Gln-Cys-	

* Amino acid no. 17 in human colipase is definitely not Cys but Asp or Glu.

Discussion

Purification of colipase from human pancreatic gland gave two colipases with different isoelectric points, but only colipase I has been fully examined. Besides colipase I and II, minor peaks with colipase activity were obtained both on DEAE-cellulose chromatography and on isoelectric focusing. The amount of colipase in peak I seems to depend on the age of the glands. A tendency to an increase in colipase II relative to colipase I was seen for glands taken at autopsy within 5–10 h compared to before this time.

Tryptic inhibitors were not added during the purification procedure as it was considered that the changes which occurred would mostly take place before the glands were removed. The low pH used for extraction was furthermore thought to prevent further proteolytic activity. The specific activity of the human colipase was lower than that of porcine origin, 25 000 compared to 40 000 $\mu\text{mol}/\text{min}$ per mg. In the assay system for human colipase porcine lipase was used and preliminary results indicate that this system has a pH optimum lower than that for porcine lipase/colipase.

Both proteins have a molecular weight of about 10 000 (a somewhat lower molecular weight for the juice colipases), without considering the contribution of the carbohydrate contents.

The N-terminal amino acid is glycine for human colipase I (gland) and colipase II (juice). Maylie et al. [1] reported the same N-terminal amino acid for porcine colipase, but Erlanson et al. [2] showed that the N-terminal pentapeptide Val-Pro-Asp-Pro-Arg is lost during the purification procedure. A similar degradation cannot be excluded for human colipase.

It is noteworthy that the amino acid methionine is present in the human and horse colipases but not in porcine and bovine colipases.

Amino acid analyses indicates that human colipase contains four disulfide bridges compared to five for the porcine, bovine and horse colipases.

Another difference is that human colipase contains carbohydrate moieties. All such residues, however, occur in smaller ratios than 1 mol per mol colipase. Such microheterogeneity is common in this type of secretion glycoprotein. The importance of this finding is not well understood [17]. Only the glucose residue is most probably an artifact (e.g., from column bed material). Glucose residues rarely appear in another species than protein (as collagen) from connective tissue (ref. 17 and Carlstedt, I., personal communication).

Kimura et al. [6], who were the first to purify a pancreatic protein activator of lipase from human pancreatic juice, reported that it contained carbohydrate and had a molecular weight of about 13 000. The carbohydrate content was reported to be 40%, but the amino acid composition, the N-terminal amino acid, the carbohydrate composition or pI were not given.

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THE PRIMARY SEQUENCE OF HUMAN PANCREATIC COLIPASE

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The amino acid sequence of an activated colipase purified from human pancreas was determined. The protein consists of a single polypeptide chain of 86 amino acids (human colipase₈₆) and has a molecular weight of 9289. The sequence was determined by automated Edman degradation of the reduced and *S*-carboxymethylated protein and of two CNBr peptides. Sequence determination of porcine procolipase II was also performed, which showed that in the original sequence determination apparently two residues were missed. These residues were determined to be a leucine at position 37 and a serine in position 50. For comparison with porcine and equine procolipases, the residues composing human colipase are numbered from 6 to 91. No human procolipase has been isolated so far. The colipases from man, pig, horse and chicken show a high degree of homology: human colipase differs from the other proteins by substitutions of 19 (porcine), 24 (equine A) and 21 (equine B) residues, respectively.

Introduction

Colipase, a small protein cofactor from the exocrine pancreas, restores the activity of lipase (EC 3.1.1.3) on triacylglycerol substrates, when these are covered with bile salts and phospholipids. For a recent review see Borgström et al. [1]. There is evidence that porcine and equine colipases are secreted in a proform [2,3], that is activated by trypsin in intestinal contents, an N-terminal pentapeptide Val-Pro-Asp-Pro-Arg being lost [4]. Trypsinolysis of procolipase also results in loss of an unknown number of amino acids at the C-terminal end. Human procolipase has not been isolated so far, possibly due to the

difficulty of obtaining fresh material. This paper describes the amino acid sequence of human colipase in activated form and discusses the homology with other colipases.

Materials and Methods

Materials

Human pancreas were collected from men (30–60 years old) who had a sudden death. Autopsies were performed within 6 h after death at the Medicolegal department, Moscow, U.S.S.R. From this raw material, human colipase was purified as described by Sternby and Borström [5]. Porcine procolipase II was purified by Dr. Anita Larsson, Lund, Sweden according to Ref. 6. Chemicals of best available grade were used. All chemicals for the Beckman sequenator was from Beckman

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Instruments Inc, Palo Alto, U.S.A., and the chemicals for the LKB solid-phase peptide sequencer was from Rathburn Chemicals Limited, Walkerburn, U.K.

Reduction and carboxymethylation of human colipase₈₆ and porcine procolipase II

The two proteins were reduced for 3 h, at room temperature under nitrogen in 0.86 M Tris-HCl (pH 8.6), 6 M Gdn-HCl, 6 mM EDTA and a 5-fold molar excess of dithioerythritol over half-cystines. Alkylation was done with iodo[2-³H]-acetic acid (Amersham, U.K.) for 15 min. The proteins were then desalted on a PD 10 column (Pharmacia, Uppsala, Sweden), eluted with distilled water and lyophilized.

Purification of CNBr fragments of human colipase₈₆

CNBr fragments of reduced and alkylated human colipase₈₆ were prepared according to Gross [7] with 50-fold molar excess of CNBr over methionines in 70% formic acid at room temperature for 24 h. The sample was diluted about 50-times with water and lyophilized.

Amino acid analysis

Amino acid analysis of the reduced and carboxymethylated human colipase₈₆ and of the CNBr peptides were performed in a Beckman 121M analyzer after 24 h and after 72 h hydrolysis in 6 M HCl/0.1% phenol at 110°C. Values for serine and threonine were derived from extrapolation to zero time, while the highest values obtained after 72 h hydrolysis were used for valine, leucine and isoleucine.

Automated Edman degradations

Edman degradations of the reduced and alkylated human colipase₈₆ and the largest cyanogen bromide fragment were done with a Beckman 890C liquid phase sequencer equipped with a cold-trap, in the presence of polybrene using a program described by Thomsen et al. [8], with a delivery of 0.2 M Quadrol before PITC presentation. The porcine procolipase II was sequenced following the same procedure.

The middle-sized cyanogen bromide fragment was immobilized on to aminopropylglass (pore size, 75 Å) via homoserinelactone [9] and se-

quenced in an LKB 4020 Solid Phase Peptide Sequencer using the standard program.

Amino acid phenylthiohydantoin were analyzed by high-pressure liquid chromatography on a column of 5 µ C-18 in sodium acetate buffer with gradient of acetonitrile. The column was operated at 37°C and the effluent was monitored at 254 nm. Phenylthiohydantoin-S-carboxymethyl-cysteine residues found were confirmed by liquid scintillation counting (not shown).

Results

Automated Edman degradation of the reduced and carboxymethylated human colipase and of the two largest (out of three) CNBr fragments was performed. The shortest CNBr fragment, glycine₆ to methionine₁₈, was elucidated earlier [5] and verified from the sequence of the reduced and alkylated protein.

N-terminal sequence of reduced and alkylated human colipase₈₆

Starting with 1 mg of S-carboxymethylated human colipase₈₆, 81 residues were sequenced with a repetitive yield of 98%. Calculations of recoveries were done for leucines in positions 34, 54 and 67, respectively, according to numbering glycine₆ to glycine₉₁.

N-terminal sequence of reduced and alkylated porcine procolipase II

Porcine procolipase II was automatically degraded according to the same procedure and successful up to residue 57.

Purification and sequence determinations of CNBr peptides

The peptide mixture resulting from chain fragmentation by CNBr was applied to Sephadex G75 superfine in 1 M acetic acid and chromatographed into four fractions as shown in Fig. 1. The amino acid composition of these fragments and the intact protein are given in Table I. Pool 0, which contained incompletely degraded material was discarded. The others designated CNBr I, CNBr II, and CNBr III corresponds to the three peptides arising from normal chain cleavage at the two methionines of the chain. CNBr I contained a

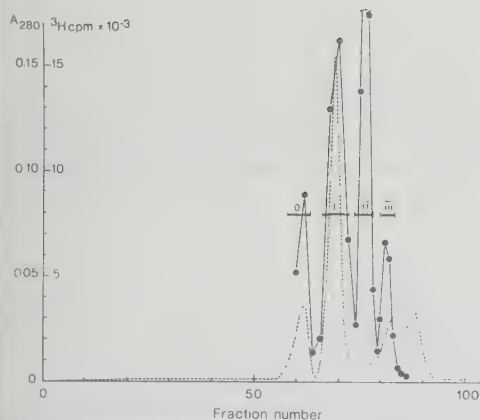


Fig. 1. Filtration through Sephadex G-75 superfine of CNBr fragments. The column ($0.8 \text{ cm}^2 \times 130 \text{ cm}$) was equilibrated and eluted with 1 M acetic acid. Flow rate 3.6 ml/h. Fraction volume 1 ml. Fractions containing CNBr I, II and III were pooled as indicated. $\bigcirc$ — $\bigcirc$, Absorbance at 280 nm. $\bullet$ — $\bullet$, Radioactivity ^3H ($\text{cpm} \times 10^{-3}$). 1/50 of each fraction was taken for scintillation counting.

TABLE I

AMINO ACID COMPOSITION OF HUMAN COLIPASE₈₆ AND THREE CNBr FRAGMENTS

Values given are expressed as moles of amino acid residue per mole of peptide. Values within parenthesis are found in the sequence. Tryptophan was not determined.

Amino acid residue	Colipase 6–91	CNBr I 43–91	CNBr II 19–42	CNBr III 6–18
Ala	4.34 (5)	1.95 (2)	2.6 (3)	(0)
Arg	2.07 (2)	1.03 (1)	0.99 (1)	(0)
Asx	8.28 (9)	4.85 (5)	1.97 (2)	1.78 (2)
Cys ^a	9.51 (10)	5.4 (5)	4.06 (4)	1.07 (1)
Glx	7.94 (8)	4.39 (4)	2.27 (2)	2.04 (2)
Gly	9.35 (9)	5.72 (6)	1.20 (1)	1.96 (2)
His	1.93 (2)	0.95 (1)	0.93 (1)	(0)
Homoserine	(0)	(0)	0.28 (1)	0.32 (1)
Ile ^b	4.89 (7)	3.52 (4)	(0)	1.58 (3)
Leu	6.01 (6)	2.10 (2)	1.91 (2)	1.77 (2)
Lys	3.93 (4)	2.9 (3)	1.07 (1)	(0)
Met	1.85 (2)	(0)	(0)	(0)
Phe	1.04 (1)	1.08 (1)	(0)	(0)
Pro	1.28 (1)	1.13 (1)	(0)	(0)
Ser	8.71 (9)	4.22 (4)	4.61 (5)	(0)
Thr	5.73 (6)	4.95 (5)	1.10 (1)	(0)
Trp	— (0)	— (0)	—	(0)
Tyr	2.71 (3)	3.08 (3)	(0)	(0)
Val	1.58 (2)	1.83 (2)	(0)	(0)
Number of residues	86	49	24	13

^a Determined as CM cysteine.

^b Too low values are obtained where Ile-Ile-Ile is found.

small amount of methionine and was redigested with CNBr and rechromatographed to become completely homogeneous. CNBr I, with 49 residues, the only peptide devoid of homoserine lactone, was considered C-terminal. This fragment was sequenced in the liquid-phase sequencer for 49 residues, which according to amino acid composition is the total amount of residues. However, the last four residues were not unambiguously determined. CNBr II, with 24 residues, was decided to be the intermediary fragment between the two methionines (Fig. 1). 75 nmol of this fragment was sequenced to the last residue in the solid-phase sequencer. Coupling yield was about 60% and the repetitive yield was 87% (determined from recoveries Ala₃ to Ala₁₅ in the peptide). CNBr III (13 residues; Fig. 1) was N-terminal and not sequenced, since its amino acid composition was identical to that of the sequence determined on the intact chain. The total number of residues found in the CNBr peptides ($13 + 24 + 49 = 86$) is in

TABLE II

AMINO-ACID SEQUENCE OF HUMAN COLIPASE₈₆

The sequence of pig procolipase II [6,10-12] with the new insertions in positions 37 and 50 and horse procolipase A and B [12,13] are shown and the horse procolipases are taken as references for residue numbering. The partial sequence of chicken colipase is also shown [14], x = residues, which are not definitely identified. Deviations from the human colipase (this investigation) are underlined. Conserved substitutions are visualized with a * on the residue. The C-terminal sequence of horse colipase A is corrected according to Dr. Catherine Chapus (personal communication). The sequence of the last four residues in human colipase₈₆ was not unambiguously determined.

	1	5	10	15	20	25
Man	GlyIle*IleIle*AsnLeuGlu*AsnGlyGluLeu*CysMetAsn*SerAlaGln					CysLysSer
Pig	<u>ValProAspProArgGlyIle</u>	IleIle	AsnLeu <u>Asp</u>	<u>GluGlyGluLeu</u>	Cys <u>Leu</u> Asn	SerAlaGln CysLysSer
Horse A	<u>ValProAspProArgGlyVal</u>	IleIle	AsnLeuGlu	<u>AlaGlyGluIle</u>	Cys <u>Leu</u> Asn	SerAla <u>Glu</u> CysLysSer
Horse B	<u>ValProAspProArgGlyVal</u>	IleIle	AsnLeuGlu	<u>AlaGlyGluIle</u>	CysMetAsn	SerAlaGln CysLysSer
Chicken	Gly <u>Leu</u> Ile <u>Phe</u> AsnLeu <u>Asp</u> <u>Thr</u> GlyGluLeu					<u>x LeuGln</u> SerAla <u>Glu</u> CysLysSer
	26	30	35	40	45	50
Man	Asn	CysCysGlnHisSerSerAlaLeuGlyLeuAla*	ArgCysThrSerMetAlaSerGluAsnSerGluCysSer			
Pig	Asn	CysCysGlnHis <u>AspThrIleLeuSerLeuLeu</u>	ArgCys <u>AlaLeuLysAlaArg</u>	GluAsnSerGluCysSer		
Horse A	<u>Glu</u>	CysCysHisGlnGluGluSerSerLeuAla	ArgCys <u>AlaAlaLysAlaArg</u>	GluAsnSerGluCysSer		
Horse B	<u>Glu</u>	CysCysHisArgGluSerSerLeuGlyLeuAla	ArgCys <u>AlaAlaLysAlaArg</u>	GluAsnSerGluCysSer		
Chicken	<u>Glu</u>	CysCysGln <u>GluAspSerGlyLeuSerLeuAla</u>	<u>x</u> Cys			
	51	55	60	65	70	75
Man	Val*LysThrLeuTyrGlyIle*	TyrTyrLysCysProCysGluArgGlyLeuThrCysGlu	Gly*AspLysThr*Ile*			
Pig	<u>Ala PheThrLeuTyrGlyVal</u>	TyrTyrLysCysProCysGluArgGlyLeuThrCysGlu	Gly	AspLysSer	<u>Leu</u>	
Horse A	<u>Ala TrpThrLeuTyrGlyVal</u>	TyrTyrLysCysProCysGluArgGlyLeuThrCysGln	<u>Val</u>	AspLysThr	<u>Leu</u>	
Horse B	<u>Ala TrpThrLeuTyrGlyVal</u>	TyrTyrLysCysProCysGluArgGlyLeuThrCysGln	<u>Val</u>	AspLysThr	<u>Leu</u>	
	76	80	85	90	95	
Man	ValGlySerIleThrAsnThrAsnPheGlyIleCysHisAspAla*	Gly*				
Pig	ValGlySerIleThrAsnThrAsnPheGlyIleCysHis <u>AsnVal</u>	Gly	<u>ArgSerAspSer</u>			
Horse A	ValGlySerIle <u>Met</u> AsnThrAsnPheGlyIleCys <u>Phe</u> AspAla	<u>Ala ArgSerGluGlxArg</u>				
Horse B	ValGlySerIle <u>Met</u> AsnThrAsnPheGlyIleCys <u>Phe</u> AspAla	<u>Ala ArgSerGluGlxArg</u>				

good agreement with the values derived from amino-acid analysis of the complete protein (Table I). An attempt to determine the C-terminal sequence of reduced and carboxymethylated human colipase with carboxypeptidase Y or carboxypeptidase A was unsuccessful. The sequence obtained for human colipase₈₆ is shown in Table II.

Discussion

The tentative primary sequence of human colipase shows that it is a single-chain protein with 86 residues, of which 10 are half-cystines. The human colipase residues are numbered after the equine procolipases. The sequence determination of porcine procolipase II showed that in the original sequence determination [10] two residues were overlooked, which were identified as a leucine in position 37 and a serine in position 50. For comparison, the sequences of 95 residues for pig procolipase II [6,1–12], 96 residues for horse procolipase A and B, [12,13] and 34 residues for chicken colipase [14] are shown in the same table. The C terminal sequence of horse colipase A is identical to the corresponding sequence in horse colipase B [12,13].

Results presented in Table II for human colipase differ from what was described earlier [5]. The previous low values of eight half cystines and 1–2 methionines can be explained by an incomplete performic acid oxidation of cysteine and methionine to cysteic acid and methionine sulphone. In the earlier sequence determination of the N-terminal of human colipase, cysteine was not found in position 17 [5]. The ten half-cystines are conserved in human, porcine II and equine A and B colipases. This fact lead to the assumption that the disulfide bridges connect the same half-cystines in all four proteins [11].

The methionine residues in human colipase are in other positions than in horse colipase. By spectrophotometric methods [12,31, the aromatic residue₅₂ (phenylalanine and tryptophan) in pig and horse colipases has been found to be of critical importance for binding of colipase to the lipid water interphase; however, human colipase has a lysine in this position.

The N-terminal part Gly₆-Ile-Ile-Ile₉ of porcine colipase is found to be important for colipase

function in the presence of phospholipids [29], but not when lipase substrates are covered only with bile salts. Also arginine₉₂ in porcine colipase is reported as essential for this binding [33].

A high degree of homology (66%) was found between human colipase, pig colipase II and horse colipases A and B for residues Gly₆-Gly₉₁. If comparison of homology is extended to involve conservative substitutions, the homology increase for all four proteins to 80%. The sequence alanine₄₃ to glycine₉₁ shows only 18 substitutions out of totally 4×49 residues for all four colipases. This almost identical part in the molecule must be of great importance for its function. The three tyrosines are conserved in the four colipases (human, porcine, equine A and B) sequenced. Many reports [15–26] propose the function of these tyrosines to be involvement in the interface recognition.

Other thoroughly investigated residues in porcine colipase [17,18,24–26] are the histidine residues, which in human and porcine colipases have positions 30 and 88. Especially His₈₈ is reported to be important, earlier numbered as 86 in porcine colipase. In horse colipases A and B the only histidine has position 29. The fact that horse colipase lacks histidine in position 88 [13] must lead to a reevaluation of the importance of this histidine [27]. Horse colipases A and B have a phenylalanine in this position.

The number of lysine residues in the interior of the colipase chain is four. Three of these are conserved in the four sequenced colipases. It has been suggested that the lysine residues in the colipase core participate in the interfacial binding of colipase [28]. The three lysines conserved has the position numbers 24, 60 and 73. The external acidic residues located in the tails have been suggested to be involved in lipase recognition [28], but only Glu₁₅ is conserved in the five sequenced colipases. In position 12 there is a conservative substitution Glu(Asp). If COO⁻ residues are modified in the porcine colipase, the ability of colipase to bind to the lipase substrate still remains, but it does not bind to lipase [28].

Comparative studies of colipase-lipase interaction [30] show that an optimal activity of a certain lipase can be obtained with different colipases. There is a tendency that lower concentration is needed of the colipase from the same species. Each

combination of colipase-lipase also has its own pH optimum [30].

The importance of homologies will become apparent when more is known about the essential residues in colipase, and the complete three-dimensional structure is solved. So far, pig and horse colipases are crystallized [12,32].

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ONE STEP PURIFICATION OF PROCOLIPASE FROM HUMAN PANCREATIC JUICE
BY IMMOBILIZED ANTIBODIES AGAINST HUMAN COLIPASE⁸⁶

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Running title: Human procolipase

Key words: Procolipase, human, pancreatic juice, immunoaffinity
chromatography

Purified antibodies to human colipase⁸⁶ were coupled to CNBr activated Sepharose 4B. The immunoabsorption column thus obtained was used to purify procolipase from human pancreatic juice in one step by immunoaffinity chromatography. A single form of procolipase was obtained, having similar biological properties as previously characterized procolipases from horse and pig. The sequence of the N-terminal propeptide was determined to be Ala-Pro-Gly-Pro-Arg. In bovine, equine and porcine procolipases the corresponding N-terminal sequence is Val-Pro-Asp-Pro-Arg.

The ability of pancreatic lipase to hydrolyze triacylglycerol substrates in the presence of bile salts and/or phospholipids is affected by pancreatic colipase (1). Colipase, a small protein cofactor, is secreted from pancreas together with lipase. Recently, evidence was obtained that porcine colipase is secreted in a proform (2), that is activated by trypsin. Trypsinolysis results in loss of an N-terminal pentapeptide and may also involve more complex C-terminal cleavage. Porcine colipase has a hundredfold higher potential to support lipolysis of complex substrates (2-4) than procolipase and is of physiological importance in the intestinal contents. Procolipase has been purified from pig (5,17), ox (6) and horse (7,16) pancreas and rat (8) and porcine (9) pancreatic juice, but so far attempts to demonstrate procolipase in human pancreatic juice or in extracts from human pancreas have failed. Colipase forms with N-terminal glycine were always obtained indicating that proteolytic cleavage may have occurred (10). The reason for this may be that it is more difficult to obtain fresh material from human sources and/or that human procolipase more easily undergoes proteolysis before or during the purification procedure.

In the present study a rapid and specific method was used to isolate procolipase from fresh human pancreatic juice based on immunoaffinity chromatography.

Preparation of antiserum to human colipase₈₆ was carried out as described previously (11). Pure antibodies were obtained by passing the antiserum through a column with human colipase₈₆ coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). Coupling of pure human colipase₈₆ to CNBr activated Sepharose 4B was done according to the method described by the manufacturer (12). Non-adsorbed material from the antiserum was washed out from the column with coupling buffer (0.1 M NaHCO₃ sodium hydrogen carbonate pH 8.3, 0.5 M NaCl sodium chloride). Pure antibodies were eluted with 0.1 M glycine-HCl pH 3. The amount of protein eluted was measured in each fraction by determination of absorbance at 280 nm. The antiserum contained about 3 mg anti-human colipase antibodies per ml. The purified antibodies were immobilized to Sepharose 4B by the method described above for human colipase₈₆. The immunoaffinity gel thus obtained was used to isolate procolipase from human pancreatic juice. Protein from the pancreatic juice, which was not adsorbed was washed out from the column with coupling buffer, see above. The adsorbed colipase was eluted with 0.5 M formic acid, 0.15 M NaCl. In each fraction from the immunoaffinity column the amount of protein and colipase was determined. Absorbance at 280 nm was used for protein determination. Colipase activity was measured using tributyrin as substrate (13). Pancreatic juice was obtained from a patient operated on for a tumour of the papilla of Vater at the Malmö General Hospital, Malmö, Sweden. The juice was drained postoperatively through a catheter and collected in a uribag at room temperature, until 50-100 ml had been obtained. Thereafter the bags were kept at -18°C until used.

Fifty ml of the frozen juice, clear and colourless, was thawed in portions and applied without any additives on the immunoabsorbent gel column at +4°C. The elution pattern is shown in Figure. Those fractions eluted with formic acid containing

colipase activity, were pooled, diluted with water and lyophilized. The colipase activity, not retained by the columns, originate from an overload of the column. The lyophilized material was dissolved in 2 x 1 ml aq. dest. and desalted on a PD10 column (Pharmacia, Sweden) in distilled water. The desalted fractions with colipase activity were pooled. In electrophoresis at pH 8.3, a colipase band appeared moving differently to human colipase forms previously obtained suggesting the presence of procolipase.

Another criteria for the presence of procolipase, a long lag time in the intralipid assay, was satisfied by the isolated colipase. At a concentration of $4 \cdot 10^{-8}$ M of human lipase and procolipase a lag time of 67 min was obtained. This value is similar to that found for the porcine procolipase/lipase system (2). Addition of trypsin to the human system reduced the lag time to 12 min.

The amino acid analysis of human procolipase was performed in a Beckman 121M analyzer after 24 h hydrolysis of the native protein in 6 M HCl at 110°C. The amino acid compositions of human procolipase and human colipase₈₆ are shown in the Table and for comparison the values for porcine procolipase I₁₀₁ are also given. The N-terminal sequence of human procolipase was determined with a Beckman 890C liquid phase sequencer, in the presence of polybrene according to Thomsen et al. (14), and it was found to be Ala-Pro-Gly-Pro-Arg. The sequence of the N-terminal pentapeptide thus differs from what is found in procolipases from other species investigated. The difference in amino acid residues between human procolipase and colipase₈₆ is bigger than can be explained by the loss of the N-terminal pentapeptide. This is probably due to the fact that trypsinolysis of procolipase also involves some splitting in the C-terminal end, that has not yet been localized. Amino acid compositions for procolipases shown in the Table indicate more amino residues (≈ 100) than is found in

the sequence analysis of porcine procolipase A, which showed 95 residues (5,18,19). Sequence data of human procolipase indicated the presence of a small amount of a colipase with an N-terminal glycine. This colipase was not detected by electrophoresis because of the small amount. This may contribute to the higher amount of residues obtained from the amino acid composition. The reason for the discrepancy in the number of Ile is that 24h is not sufficient for total hydrolysis of the IleIleIle bonds in the N-terminal. The amino acid composition will therefore give too low a value for Ile.

Immobilized antiporcine procolipase antibodies were previously used in an attempt to isolate procolipase from human pancreatic juice (9) but two activated forms with an N-terminal glycine were obtained. Two activated forms were also seen if the juice was left at room temperature. These colipase forms from juice thus obtained correspond on electrophoresis (pH 8.3) with colipase₈₆ (15) and colipase ≥ 87 respectively, from human pancreatic gland (B. Sternby, unpublished).

Antihuman colipase antibodies cross react with all colipases investigated, when ELISA technique was used (11). The binding of antihuman colipase antibodies might be stronger to human than to nonhuman colipases. With antiporcine procolipase antibodies Rathelot et al. (17) found slight cross reactivity to human colipase, and none to bovine, canine, equine and chicken colipases. The sequence similarities of human, equine and porcine colipases (15) do not indicate the existence of antigenic determinants in one colipase, which are not detectable by antibodies to another colipase. The reported differences might be the result of the different antigens used (human colipase₈₆ and porcine procolipase, respectively) and/or different immunological techniques (ELISA versus precipitation methods). The previous diffi-

culty in obtaining human procolipase may be explained by a more efficient proteolysis caused by the different N-terminal sequence.

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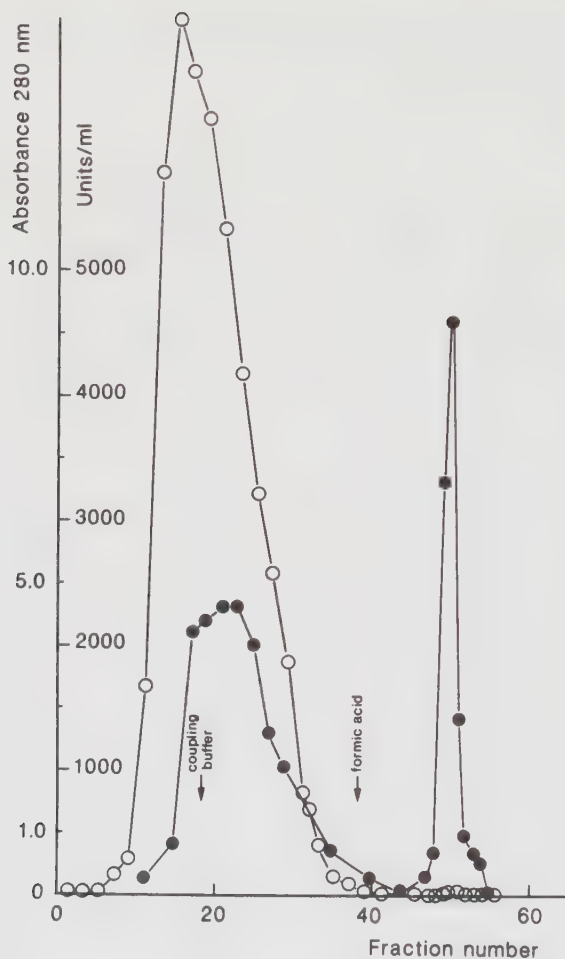
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Table 1. Amino acid composition of human colipase (16), human procolipase and porcine procolipase I₁₀₁ (Dr. Anita Larsson, Lund, Sweden, unpublished).

	Human colipase	Procolipases	
		Human	Porcine
Asx	8.28 (9)*	10.81	12.20
Thr	5.73 (6)	8.09	5.45
Ser	8.71 (9)	8.91	9.07
Glx	7.94 (8)	9.18	9.43
Pro	1.28 (1)	4.56	4.67
Gly	9.35 (9)	10.96	8.36
Ala	4.34 (5)	6.00	5.44
1/2 Cys**	9.51 (10)*	10*	10*
Val	1.58 (2)	3.58	4.22
Met	1.85 (2)	2*	0.00
Ile	4.87 (7)	5.40	6.00
Leu	6.01 (6)	7.29	9.64
Tyr	2.71 (3)	3.87	3.61
Phe	1.04 (1)	1.70	2.11
His	1.93 (2)	1.93	2.00
Lys	3.93 (4)	4.50	4.16
Arg	2.07 (2)	3.37	5.66
Total number of residues	81.14 (86)	102.15	102.20

*Number of aminoacid residues found in the sequence.

**Determined as CM cysteine.



LEGEND TO FIGURE

Elution pattern for isolation of colipase from human pancreatic juice by immunoaffinity chromatography (for details, see text).

Volume of each fraction, 2.8 ml

Colipase activity.

Total proteins, determined as absorbance at 280 nm.

PURIFICATION AND CHARACTERIZATION OF PANCREATIC COLIPASE FROM
THE DOGFISH (SQUALUS ACANTHIUS)

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SUMMARY

Pure colipase from dogfish (*Squalus acanthius*) was obtained from an extract of pancreatic gland. It has a high isoelectric point (10.2) and the molecular weight was calculated to be 9108-9383. The N-terminal sequence was shown to be Gly-Leu-Phe-Leu-Asn-Leu-Ser-Ala-Gly-Glu-Leu-Cys-Val-Gly-Ser-Phe-Gln-Cys-Lys-Ser-Ser-Cys-Cys-Gln-Arg-Glu-Thr-Gly-Leu-Ser-Leu-Ala-Arg-Cys-Ala-. This sequence shows great homology with colipases from man, horse, pig and hen. There were indications of the existence of a proform of dogfish colipase. The propeptide was found to be Ala-Pro-Glu-Arg.

INTRODUCTION

Colipase, an acid- and heat-stable polypeptide of low molecular weight, is found with lipase in exocrine pancreatic secretion. Inhibition of pancreatic lipase by bile salt is specifically reversed by colipase (1). Pancreatic colipase has been purified from various sources such as pig (2,3,4), ox (5), horse (6), man (7) and hen (8). To date, most of the structural and functional investigations of colipase have been carried out on the porcine, equine, and human proteins (9-12).

Evidence for colipase in fish pancreas has been reported for dogfish (13,14), trout (15), hagfish, ratfish, rayfish and Greenland shark (14). The complete amino acid sequence of colipase has been established for the pig (9), horse (10,11) and human (12) colipases. The N-terminal sequence of 34 residues has been determined for the chicken colipase (8). No colipase from fish pancreas has previously been isolated. In this study the purification and some properties of a fish colipase are reported. The N-terminal amino acid sequence of dogfish colipase is compared with the five avian and mammalian colipases so far investigated.

MATERIALS AND METHODS

Source of colipase. About 150 pancreatic glands were removed from freshly killed dogfish at Kristineberg Marine Biology Station, Fiskebäckskil, Sweden. After removal, the glands were kept on dry ice and then stored at -80°C until use.

Determination of colipase. Colipase was assayed at room temperature ($20-23^{\circ}\text{C}$) by the potentiometric titration method (16). Assays were performed at pH 7.0 with tributyrin as substrate in

the presence of 2 mM sodium taurodeoxycholate (17) and an excess of pure human lipase. One unit of colipase activity corresponds to one micromole butyric acid released per minute under standard conditions.

Determination of protein. Protein concentration was determined spectrophotometrically at 280 nm and $A_{280}^{1\%}$ was chosen as 10.0.

Amino acid analysis. The amino acid composition of colipase after acid hydrolysis in 6 M HCl for 24 h and 72 h at 110°C under vacuum was determined with a Beckman 121M amino acid analyser. The cysteine and methionine contents were determined after performic oxidation.

Preparation of reduced and carboxymethylated (RCM) colipase. The dogfish colipase was reduced and S-carboxymethylated as earlier described for human colipase (12).

Amino terminal sequence. Sequence analysis was carried out on both the unreduced activated protein and a mixture of reduced and carboxymethylated proform and activated form. A Beckman 890C Sequenator was used for these studies as earlier described for human colipase (12).

Determination of the molecular weight. An approximate molecular weight was obtained by gel chromatography with human colipase as standard. A probable value of the molecular weight was calculated from the amino acid analyses according to Delaage (18).

Isoelectric focussing. The isoelectric point was determined with a 1% ampholine gradient (pH 7-10.5) (LKB Produkter, Sweden). Sucrose was used as gradient stabilizer and the method used was that described by Vesterberg et al. (19).

Purification procedure. The first step in the purification of dogfish colipase was made according to a description originally designed to isolate hagfish insulin (20). All side fractions were saved for this purpose during this purification procedure. The frozen pancreatic glands (160 g) were thus homogenized, centrifuged, and prepared as described (20) and an air-dried diethyl-ether precipitate was obtained.

The precipitate was dissolved in 25 ml of 3 M acetic acid (HAc) and divided into two portions. Each portion was applied to a glass column (5.0 x 100 cm) containing Biogel P-30 in 3 M HAc. The colipase was eluted with 3 M HAc and the fractions with colipase activity were pooled. The acetic acid was evaporated with a rotary evaporator and the protein redissolved in 50 ml of distilled water. The pH of this solution was 4.0. The sample was applied to a SP Sephadex C-25 column (0.9 x 15 cm) equilibrated with 10 mM sodium acetate buffer pH 5.0 and washed with 100 ml of 0.01 M NH_4HCO_3 pH 8.0. The colipase was eluted with a gradient (2 x 50 ml) 0.01-0.5 M NH_4HCO_3 , pH 8.0. The fractions with colipase activity were pooled, dialyzed and lyophilized. After gel filtration on Sephadex G-50 Superfine in 0.1 M NH_4HCO_3 , pH 8.0 the colipase was homogeneous both in electrophoresis at pH 4.3 and in SDS-PAGE (see below). The G50 fractions with colipase activity were pooled, dialyzed and lyophilized and used for further analyses.

Polyacrylamide gel electrophoresis. Gel electrophoresis was performed at pH 4.3 (alanine-HCl buffer) in 15% polyacrylamide. Gels were stained with Coomassie brilliant blue (21) and destained in HAc:MeOH:H₂O, 7:25:68 (v:v:v).

SDS electrophoresis. SDS electrophoresis was performed at pH 9.18 in 12% polyacrylamide calibrated with standard proteins from the "low molecular weight kit" (Pharmacia, Sweden).

RESULTS

The overall purification of dogfish colipase from 150 pancreatic glands (160 g) is summarized in Table I. The yield of colipase was about 10% of the activity originally present in the extract of dogfish pancreas. Homogeneity of the preparations was confirmed by polyacrylamide gel electrophoresis and SDS gel electrophoresis.

Amino acid composition. The amino acid composition of colipase from dogfish pancreas is given in Table II. The compositions of human, porcine, horse and chicken colipases are also given for comparison.

Isoelectric focussing. The isoelectric point of dog fish colipase was found to be 10.2.

N-terminal sequence. Table III shows the amino acid sequence of the amino terminal part of dogfish colipase. A comparison with the sequences of human, pig, horse and chicken cofactors is also presented. The main part of dogfish colipase was obtained in a form with a glycine as N-terminal amino acid, and the proform was not demonstrated until result from the sequence determination of the RCM colipase was obtained. The N-terminal propeptide appeared to be a neutrally charge tetrapeptide Ala-Pro-Glu-Arg.

DISCUSSION

Elucidation of the amino terminal sequence of dogfish colipase allows for the first time a comparison of the partial structure of a fish colipase with one avian and four mammalian colipases. As shown in Table III, dogfish procolipase does not possess the N-terminal pentapeptide but a tetrapeptide Ala-Pro-Glu-Arg. In

man the propentapeptide is Ala-Pro-Gly-Pro-Arg (27) and in horse and pig, Val-Pro-Asp-Pro-Arg. The sample, which by sequence studies was found to contain two forms of dogfish colipase was homogeneous on electrophoresis. Even if this sequence determination is the only proof for a dogfish procolipase we propose that the colipase exists as a proform. Whether the proform was activated before or during purification or this activation occurred during the preparation of a RCM colipase is not known.

Under the conditions used in colipase purification, the propeptide may be lost by proteolytic cleavage (3). In the present study colipase was purified from tissue homogenate in acidic 80% ethanol and under similar conditions pig and horse procolipases were found, but chicken colipase (8) was only purified in activated form. It has been shown by Borgström et al. (22,23) that removal of this propeptide increases the binding of colipase to a phospholipid-stabilized emulsion.

The dogfish colipase has a high isoelectric point, 10.2, compared to other colipases (28), which vary between 5.3-7.9. Comparing the presented sequence of the dogfish colipase to the corresponding part of the avian and mammalian colipases, one can observe a large degree of sequence homology. Of the 35 residues 16 are identical, and residues at 3 of the 19 remaining positions are related by single conserved amino acid substitutions. It has previously been suggested that the N-terminal region of the colipase molecule might be part of the site of interaction with lipase (24). The observed similarities in the sequences support the idea that this region is important for the function of the cofactor.

Although the sequence reported herein is partial it appears that variations between fish, mammalian and avian colipases are of the same order of magnitude as those observed among mammalian cofac-

tors, the percentage of amino acids identical in dogfish and human colipases being 57 and in dogfish and chicken colipases 64. If conserved substitutions are considered these values increase to 68% (man) and 75% (chicken).

The lower specific activity obtained with human lipase/dogfish colipase (5600 compared to 25,000 for the pure human system) (7) can be explained by the differences in the colipase molecules such as the very different pI (pI=5.8 for human colipase₈₆).

Of interest are the immunological studies of dogfish colipase with antibodies to human colipase. With the ELISA technique it was shown that antibodies to human and porcine colipases cross-react with colipase from dogfish (14). Radioimmunological assay of human pancreatic colipase₈₆ (unpublished) shows reactivity with the dogfish colipase, but with lower affinity than for the human colipase. The very high degree of homology between colipases is also verified by immunoadsorption of colipases (e.g. rat, hen, pig and hagfish) from unpure preparations to antibodies against human colipase coupled to CNBr Sepharose 4B. With antibodies to porcine procolipase B, however, Rathelot et al. (25) showed that these antibodies cross react with all types of porcine procolipases and colipases and partly with human colipase, but not with chicken, ox, horse or dog colipases. A possible explanation for the observed discrepancies in results could be the different immunological techniques used e.g. radioimmunoassays without addition of PEG 6000, which is of importance in detecting small antigens like colipase. However, it could also be due to the fact that the antibodies used were directed against different antigenic determinants. ELISA is more sensitive (26) and the method introduces no need for precipitated colipase and antibody complex to measure the cross reactivity.

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TABLE I

Purification of dogfish colipase from pancreatic gland.

Purification step	Colipase activity units	Proteins mg	Specific activity units/mg	Yield
Supernatant before pH 8.5 (610 ml)	289,000			100%
Before Biogel (25 ml)	150,000	677	220	52%
Before SP-Sephadex (100 ml)	43,000	111	430	15%
G50	22,000	3.7	5600	10%

TABLE II

Amino acid composition of dogfish pancreatic colipase and comparison with colipase from man, pig, horse and chicken.

Amino acid	Residues per molecule				
	Dogfish	Man (12)	Pig (9)	Chicken (8)	Horse (10)
Ala	4 (4.34)	5	4	6	9
Arg	3 (3.15)	2	5	4	5
Asx	10 (9.73)	9	12	11	8
Cys	9-10 (9.44)	10	10	9-10	10
Glx	9-10 (9.52)	11	6	8	13
Gly	9 (8.86)	9	10	9	6
His	1 (1.03)	2	2	0-1	1
Ile	3 (3.01)	6	5	3	5
Leu	9 (8.84)	6	10	8	7
Lys	7 (6.80)	4	4	5	4
Met	1 (1.01)	2	0	0-1	1
Phe	3 (2.91)	1	2	1	2
Pro	3 (2.98)	1	3	2	3
Ser	8 (7.92)	9	8	10	9
Thr	2-3 (2.47)	6	5	5	4
Trp	not determined	0	0	0	1
Tyr	3 (3.03)	3	3	4-5	3
Val	2-3 (2.56)	2	4	4	5
Total number					
of residues	86-90	86	93	89-93	96

TABLE III

Partial amino acid sequence of procolipase from dogfish pancreas. The sequences are arranged to obtain maximal homology. The corresponding sequences for man (12,27), pig (3,9), horse A and B (10,11) procolipases and chicken (8) colipase are shown. x=Residues which are not definitely identified. (-)=Residue which is deleted. Deviations from the dogfish colipase (this investigation) are underlined. Conserved substitutions are visualized with * on the residue.

	1	5	10	15	20
Dogfish	Ala [*] ProGlu - ArgGlyLeu [*] PheLeuAsnLeuSerAlaGlyGluLeu [*] CysValGlySer				
Man	AlaProGlyProArgGlyIle	IleIleAsnLeuGluAsnGlyGluLeu	CysMetAsnSer		
Pig	ValProAspProArgGlyIle	IleIleAsnLeuAspGluGlyGluLeu	CysLeuAsnSer		
Horse A	ValProAspProArgGlyVal	IleIleAsnLeuGluAlaGlyGluIle	CysLeuAsnSer		
Horse B	ValProAspProArgGlyVal	IleIleAsnLeuGluAlaGlyGluIle	CysMetAsnSer		
Chicken		GlyLeu	IlePheAsnLeuAspThrGlyGluLeu	x	LeuGlnSer
	21	25	30	35	40
Dogfish	PheGlnCysLysSerSerCysCysGlnArgGluThrGlyLeuSerLeuAla [*] ArgCysAla				
Man	AlaGlnCysLysSerAsnCysCysGlnHisSerSerAlaLeuGlyLeuAla	ArgCysThr			
Pig	AlaGlnCysLysSerAsnCysCysGlnHisAspThrIleLeuSerLeuLeu	ArgCysAla			
Horse A	AlaGluCysLysSerGluCysCysHisGlnGluGluSerLeuSerLeuAla	ArgCysAla			
Horse B	AlaGlnCysLysSerGluCysCysHisArgGluSerSerLeuGlyLeuAla	ArgCysAla			
Chicken	AlaGlnCysLysSerGluCysCysGlnGluAspSerGlyLeuSerLeuAla	x	Cysx		

EVOLUTIONARY STUDIES ON PANCREATIC COLIPASE

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In this evolutionary study the following criteria have been used to prove the existence of colipase: 1. It restores the activity of human and porcine pancreatic lipase inhibited by bile salt. 2. It cross-reacts with antisera to human and porcine colipases. 3. Its restoration of lipase activity, inhibited by bile salt, in the tributyrin assay system, is prevented by antiserum to colipase. 4. It is a heat-stable, low-molecular-weight protein (molecular weight about 10 000 by gel-filtration). The occurrence of colipase has been verified in the exocrine pancreatic cells from hagfish (*Myxine glutinosa*), ratfish (*Chimaera monstrosa*), rayfish (*Raja radiata*), Greenland shark (*Somnius microcephalus*) and dogfish (*Squalus acanthius*). No colipase activity could be found in the gastric juice of crayfish (*Pacifastacus leniusculus*). These results indicate that colipase evolved in the vertebrates before the organized exocrine pancreatic gland and occurred simultaneously with the bile salts/bile alcohols.

Introduction

Colipase is a low-molecular-weight protein secreted by the exocrine pancreas. Its function is to restore the activity of pancreatic lipase in the presence of bile salts [1]. It has been isolated from the pancreas of many mammals (man, dog, horse, ox, pig, rat and sheep) [2–8] and from the chicken [9]. The amino acid composition and sequence seems to be rather conserved in these species. Colipase activity has been demonstrated in the extract of pancreas from shark and trout [10,11]. In general, colipase from one species, seems to be able to interact with lipase from other species [12]. To study the evolution further we have now looked for colipase in the intestinal tract from some lower vertebrates and one invertebrate, the crayfish. Except for the hagfish, the vertebrates studied have a well-organized exocrine pancreas. The hagfish has exocrine pancreatic cells diffused around the gut lumen [13]. The crayfish has a hepatopancreas around the lumen of the intestinal tract. It also

differs from the other species by not producing bile salts or bile alcohols but a detergent of the acylpeptide type [14].

Materials and Methods

Chemicals

Sephadex G-100 coarse was obtained from Pharmacia, Uppsala, Sweden. Sodium taurodeoxycholate was prepared from deoxycholic acid, using the mixed anhydride method [15]. Tributyrilglycerol was a product of B.D.H. Chemicals, Poole, U.K. Microtitre plates were purchased from NUNC, Denmark and alkaline phosphatase conjugated swine antibodies against rabbit IgG from Orion diagnostica, Helsinki, Finland. Bovine serum albumin fraction V powder and *p*-nitrophenylphosphate, Sigma 104 phosphate substrate tablets were products of Sigma.

Assay of colipase

Colipase activity was determined using tri-

butyryl as substrate, as described [6]. 1 colipase unit expresses the number of μmol of butyric acid released per min in the assay system. The specific activities of human and porcine colipases with this substrate are 25 000 units/mg [2] and 40 000 units/mg [6], respectively.

The amount of colipase present was related to its activity at pH 7.0 with an excess of human lipase. It was assumed that 1 mg of a pure protein corresponds to 25 000 units.

Preparation of antisera to human and porcine colipases

Antisera to activated human and porcine colipases were raised in rabbits. On day 1 the rabbits were injected subcutaneously in the neck with 1 mg colipase dissolved in 400 μl 0.15 M NaCl and 600 μl Freund's complete adjuvant. Booster doses with the same amount of antigen in Freund's incomplete adjuvant were given after 4 weeks and then every second week until the antibody titres were constant. The rabbits were bled 10–12 days after the last booster and then every 2 weeks. The immunization continued until enough serum was obtained. The amount of specific antibodies in the respective antiserum was calculated to be about 0.3 mg/ml. To eliminate the blood cells, the antisera were centrifuged (room temperature, 1500 rpm, 10 min). The antisera were kept at -20°C until use.

Inhibition of colipase activity by antiserum to colipase

Colipase (20 pmol) and antiserum to colipase (1–20 μl) were incubated in 15 ml of buffer containing 2 mM sodium taurodeoxycholate for 2 min [6]. Tributyrin (0.5 ml) and lipase (60 pmol) were then added. The activity was measured and the inhibition calculated as percentage of activity without antiserum. Nonspecific rabbit serum was used as control. Antiserum was also added after the tributyrin and lipase.

Heat-stability test

The heat stability of colipase was checked by keeping the solution with colipase activity at 70°C for 10 min.

Gastric content of crayfish

Gastric content of crayfish was sucked up from

the stomach with a pasteur pipett. The fluid was put in a beaker on dry ice. The crayfish were obtained from Simontorp, Skåne, Sweden.

Colipase from hagfish

Colipase was obtained both from the intestinal wall and intestinal content of hagfish. Intestines (44 g) from about 20 deep-frozen specimens were cut out and homogenized in 0.25 M sucrose to a 10% solution. The homogenate was centrifuged for 30 min at 9000 rpm at 4°C . The intestinal content (2 ml) was diluted to 5 ml with 0.25 M sucrose. These two samples were each chromatographed on a Sephadex G100 coarse column equilibrated in a buffer containing 2 mM Tris-maleate, 1 mM CaCl_2 , 150 mM NaCl and 4 mM sodium taurodeoxycholate, pH 7.0 at room temperature ($20\text{--}23^{\circ}\text{C}$). Two peaks with colipase activity were obtained, one in the void volume and one in an elution volume with a K_{av} value of about 0.5 [17]. The low-molecular-weight peak (K_{av} 0.5) was used as the colipase source.

Colipase from ratfish, rayfish and Greenland shark

Gel-filtration (see above) of a 10% homogenate of pancreas in 0.25 M sucrose gave the colipase from ratfish (nine glands, totally 1.8 g), rayfish (1.4 g) and Greenland shark (33 g).

Colipase from dogfish, man and pig

Pure colipase from dogfish was isolated according to the method of Sternby et al. (unpublished data). In short, the method used was the following. The frozen dogfish glands were homogenized in acidic ethanol at 4°C . The homogenate was centrifuged, the pellet discarded and the pH of the supernatant brought to 8.5. The precipitate obtained here was rejected after another centrifugation. The supernatant after this procedure was precipitated with ethanol and diethyl ether. This precipitate was redissolved in 3 M HAc and after a gel chromatography step and ion-exchange chromatography the pure dogfish colipase was obtained. Colipases with N-terminal glycine and lipases from man [2,12] and pig [6,19] were purified as described.

ELISA (enzyme-linked immunosorbent assay)

The ELISA technique described by Engvall and

Perlmann [16] was used to check the direct binding between colipase and antibodies. Microtiter plates were coated with 200 μ l colipase at a concentration of 25–100 ng/ml in 0.05 M Na_2CO_3 , pH 9.6, for 16–20 h at room temperature. After four washes with 0.15 M NaCl containing 0.05% Tween 20, the plates were incubated for 1 h at room temperature with 200 μ l antiserum diluted 1:100 with an incubation buffer (8 g NaCl/1.425 g $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ /0.2 g NaN_3 /0.5 ml Tween 20/0.2 g KCl/0.2 g KH_2PO_4 per l, pH 7.4). The plates were then washed four times as above and 200 μ l of alkaline phosphatase conjugated swine antibodies against rabbit IgG (diluted 1/1000 with incubation buffer (see above) containing 2 g bovine serum albumin/l) was added. The plates were left for 16–20 h at 4°C. The final washings were followed by addition of 200 μ l of the substrate *p*-nitrophenylphosphate 1 mg/ml in a buffer containing 1 M diethanolamine and 0.5 mM MgCl_2 pH 9.8. The plates were incubated at room temperature for 1 h. The amount of *p*-nitrophenylphosphate hydrolyzed is proportional to the amount of colipase. Released *p*-nitrophenol was measured at 405 nm in a Multiscan Spectrophotometer, Flow Laboratories, McLean, VA, U.S.A.

ELISA was also used to measure the capacity of each colipase to inhibit the binding between human colipase and antibodies to human colipase. A fixed concentration of antiserum (1:100) to human colipase was preincubated with different concentrations (1–100 ng/ml) of the colipase of interest. Thereafter, the remaining unbound antibodies were transferred to a microtiter plate precoated with a fixed amount of human colipase. The amount of human colipase-antibody complex formed was measured as above.

Controls

Each series of colipase samples contained wells which received only buffer instead of colipase or colipase with an incorrect antiserum 1:100, e.g., to human pancreatic lipase or phospholipase A_2 instead of colipase. These controls generally gave absorbance values under 0.1. Each antiserum was assayed in duplicate and each colipase concentration in triplicate for each serum.

Results

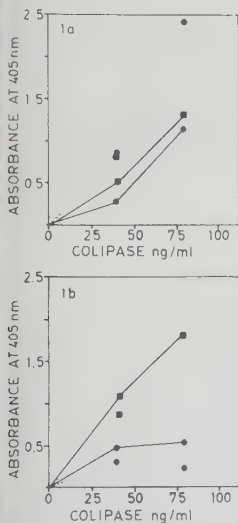
The proof for the presence of colipase was: 1. Colipase has the ability to restore bile salt-depressed human and porcine lipase activity. 2. Antisera to human and porcine colipases cross-react with colipase from lower vertebrates. 3. Colipase activity is specifically depressed by antiserum to colipase. 4. Gel-filtration of colipase gives a molecular weight of about 10000. 5. Colipase keeps its activity even after being held at 70°C for 10 min.

According to these criteria, no indication for colipase was obtained in gastric content from crayfish (invertebrate). The proofs for colipase were all fulfilled by extracts from hagfish, ratfish, rayfish, Greenland shark and dogfish. Only qualitative aspects were considered because of the small amount of starting material which was available. Accordingly, the colipase amount/activity was low, but its specific activity high. The restoration of lipase activity was thus not an effect of any non-specific protein. ELISA was used to measure the cross-reactivity between antibodies to human and porcine colipases, respectively, and the different colipases from the lower vertebrates. The cross-reactivity was measured both as direct interaction of colipase and antibodies (Figs. 1a, b) and as capacity of colipase to inhibit the binding between human colipase and antibodies to human colipase (Fig. 2). Colipase activity was inhibited by antiserum to human colipase if colipase and antibodies were incubated (2 min) before the addition of tributyrin and lipase. Nonspecific serum had only a small effect in this system. Specific antiserum had about the same effect as nonspecific serum, when added after tributyrin and lipase (Fig. 3). The shape of the inhibition curves were very similar for the different colipases with antisera to human or porcine colipases.

Human colipase was used as a standard for determination of an approximate molecular weight by gel filtration. Gel chromatography of the different extracts gave K_{av} values of about 0.5 corresponding to a molecular weight of approximately 10000, as found for human and other colipase investigated [2–8]. Colipase activity also was found in the void volume peak corresponding to a macromolecular aggregate of colipase, proteins and lipids. These also contained lipase activity against

tributyryn (lipase rapid [29–33]).

The void complex keeps its colipase activity after heat inactivation, but loses its lipase activity. By



Figs. 1a,b. Cross-reactivity of anti-human (1:100, —) and anti-porcine (1:100, ---) colipase antiserum and colipases from hagfish intestine (●) and rayfish (■) (a), and rayfish (●) and Greenland shark (■) (b) measured as direct binding between colipase and antibodies. For explanation, see text.

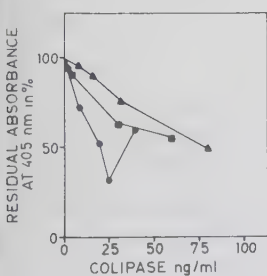


Fig. 2. Cross-reactivity of anti-human colipase antiserum and colipases from hagfish (intestine) (●), rayfish (■) and Greenland shark (▲) measured as the capacity of each colipase to inhibit the binding between human colipase (20 ng/ml) and antibodies to human colipase (1:100). For explanation, see text.

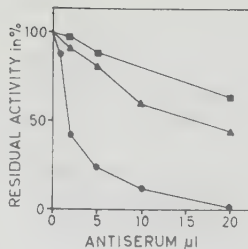


Fig. 3. Inactivation of colipase from dogfish with antiserum to human colipase. Experiments carried out with specific (●) and nonspecific (■) serum. Specific antiserum (▲) was added after tributyrin and lipase. For explanation, see text.

lipase activity in the void peak is meant activity in bile salt solution against tributyrin. This lipase activity can originate in pancreatic bile salt-dependent lipase, which has been reported in several species [22–24] and/or pancreatic lipase in the presence of colipase. No difference was found in the ability of the colipases to restore bile salt-depressed human or porcine lipase activities. Nor was any difference found between the antisera to human and porcine colipases in the cross-reaction with the colipases from the lower vertebrates.

Discussion

The results above indicate that the colipase molecule is as old as the bile salts/alcohols and older than the well-organized pancreas. Colipase is thus not a newcomer among pancreatic proteins. This result contradicts the suggestion that a bile salt-dependent lipase would play the role of the colipase-lipase-bile salt system in lower animals. Patton et al. [20] have reported that leopard shark has only this bile salt-dependent lipase. But they used a crude pancreatic sample as enzyme source and no attempt was reported in which the possibility that colipase-lipase activity was present in the bile salt-dependent lipase activity could be definitely ruled out.

We found no indication for a colipase in gastric content from crayfish that activated human lipase. Brockerhoff [22], on the other hand, found that the digestive lipase of lobster in a purified form did not hydrolyse tributyrin unless a protein

cofactor was present. This requirement was non-specific and could be replaced by bovine serum albumin. Invertebrates do not possess a true liver and do not secrete a separate digestive fluid that could be described as bile. But they do have a hepatopancreas and surface-active compounds of the acyl peptide type in their alimentary tracts, which depress the activity of porcine pancreatic lipase [21]. Our finding does not exclude the possibility of the existence of a colipase-like molecule in the invertebrates, but it does not fulfill our criteria of colipase. Bile salts are produced by the liver in all vertebrates so far investigated. In the hagfish (*Myxinoidea*) at the lowest level of organization among true vertebrates, a large gallbladder and ample bile were found. No vertebrates below birds and mammals have so far been found to lack a gallbladder. Hagfish lacks a well-organized pancreas, and in this material the lowest amount of colipase activity was obtained. Pancreatic proteases such as trypsin and chymotrypsin [13] and insulin [18] have been identified or isolated from the hagfish intestine. The existence of colipase in the lower vertebrates reported in this paper is no proof that there is also pancreatic lipase. The tiny amount of material, and the lack of antiserum to pancreatic lipase at that time made it impossible to determine the nature of the lipolytic activity in the void volume, i.e., bile salt-dependent lipase, and/or lipase and colipase. Bile salt-dependent lipase [20] is probably the same enzyme as pancreatic non-specific lipase [23,24] pancreatic carboxylic esterase [25,26] and pancreatic cholesterol esterase [27,28]. This enzyme has wide substrate specificity and an absolute demand for trihydroxy bile salts.

Patton et al. [20] reported that there is only a bile salt-dependent lipase in the pancreas of the leopard shark. They noticed, that this lipase activity increased 4-fold when pure sodium taurocholate was changed to native bile. Possibly the colipase-lipase activity was hidden under the bile salt-dependent lipase activity or not maximal in the absence of other bile salts than sodium taurocholate.

The colipase-lipase complex, which is obtained from crude pancreatic preparations on gel filtration, behaves like a bile-salt dependent lipase.

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COMPARATIVE STUDIES ON THE ABILITY OF PANCREATIC COLIPASES TO RESTORE ACTIVITY OF LIPASES FROM DIFFERENT SPECIES

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Abstract—1. It is reported that pancreatic colipase from various sources—dog, man, pig and rat—restores the activity of bile salt-inhibited lipase from the same species in all combinations.

2. Colipase therefore shows no species specificity, contrary to the results of a recent report indicating that canine colipase did not stimulate the lipolytic activity of bile salt-inhibited lipase from human and rat pancreas.

INTRODUCTION

Pancreatic lipase needs a cofactor, colipase, in order to retain full lipolytic activity in the presence of physiological concentrations of bile salts. This has been shown by several groups (Baskys *et al.*, 1963; Morgan *et al.*, 1969; Maylie *et al.*, 1971; Morgan & Hoffman, 1971; Borgström & Erlanson, 1971). Colipase has been found in the pancreatic gland and/or pancreatic juice of cow, dog, fish, horse, man, pig, rat and sheep (Baskys *et al.*, 1963; Morgan *et al.*, 1969; Maylie *et al.*, 1971; Morgan & Hoffman, 1971; Borgström & Erlanson, 1971; Julien *et al.*, 1972; Kimura *et al.*, 1974; Canioni *et al.*, 1975; Lee, 1978; Patton *et al.*, 1978; Sternby & Borgström, 1979; Leger *et al.*, 1979). Published reports are contradictory regarding species specificity between colipase and lipase. Colipase has been claimed to be a universal cofactor for pancreatic lipase by Canioni *et al.* (1975), Vandermeers *et al.* (1974, 1975), Rathelot *et al.* (1975), Leger *et al.* (1979). Lee (1978) states that canine colipase activates bovine, canine and porcine lipase but not that of man and rat. The present study agrees with the statement that colipase is a universal activator for pancreatic lipases.

MATERIALS AND METHODS

Partial purification of colipase and lipase from canine pancreatic juice

Canine colipase and canine lipase were separated by gel filtration in 50 mM sodium acetate buffer pH 5.0 using Sephadex G-75 (Pharmacia, Sweden).

Purification of colipase and lipase from human pancreatic gland

Human colipase I was purified according to the procedure described by Sternby & Borgström (1979).

Human lipase was purified from pancreatic glands, which were obtained at autopsy within 10 hr of death. The glands were minced, delipidated, centrifuged and fractionated using ammonium sulfate as for human colipase (see above). The first Sephadex G-100 chromatography gave one peak with lipase activity and one with colipase activity.

The lipase peak was pooled and dialysed against 10 mM Tris-HCl buffer, pH 8.0 overnight and then put on a DEAE-cellulose column in the same buffer. The lipase activity, which did not bind to the column, was dialysed against 10 mM Tris-succinate buffer, pH 6.3. The dialysed lipase was then put on a CM-cellulose column in the same buffer and eluted with a sodium chloride gradient (0.0–0.3 M). Two partially separated peaks of lipase activity were obtained, with the same specific activity $\approx 8000 \mu\text{mol/min/mg}$ protein, with glyceroltributyrate (BDH) as substrate.

Purification of colipase and lipase from porcine pancreatic gland

Porcine colipase and lipase were purified as previously described (Erlanson & Borgström, 1972; Donné, 1976).

Partial purification of colipase and lipase from rat pancreatic juice

Rat colipase was obtained by Sephadex G-100 filtration of rat pancreatic juice. Fractions with colipase activity were pooled, lyophilized and redissolved in a small amount of lipase buffer (see below). Rat lipase was purified as described by Gidez (1968).

Assay for colipase and lipase

Colipase and lipase activities were determined as described by Erlanson & Borgström (1972) using glyceroltributyrate as substrate and with a surplus of lipase (20 pmol). Colipase and lipase activities were determined at room temperature when the rate of hydrolysis was in a steady state. Colipase and lipase activities were also determined as described by Vogel & Zieve (1963) except that the substrate was sonicated using a Branson Sonifier Model S 123. The buffer was 50 mM Tris-HCl pH 8.8, 0.35% sodium deoxycholate and olive oil. To 50 ml of this buffer 20 μl olive oil was added and the solution was sonicated for a few minutes. The optical density of this emulsion at 500 nm was about 1.3 (1 cm cuvette length). The emulsion was stable for at least a day and 1 ml was used for each experiment. The activity was measured as the decrease in absorbance at 500 nm. The amount of colipase in volumes of 2–20 μl was the same in both methods. Twenty pmoles of lipase in volumes of 10–20 μl were used in the titration method and half of this amount with the method of Vogel & Zieve (1963) to enable the reaction to proceed for at least 10 min for comparison with the results of Lee (1978).

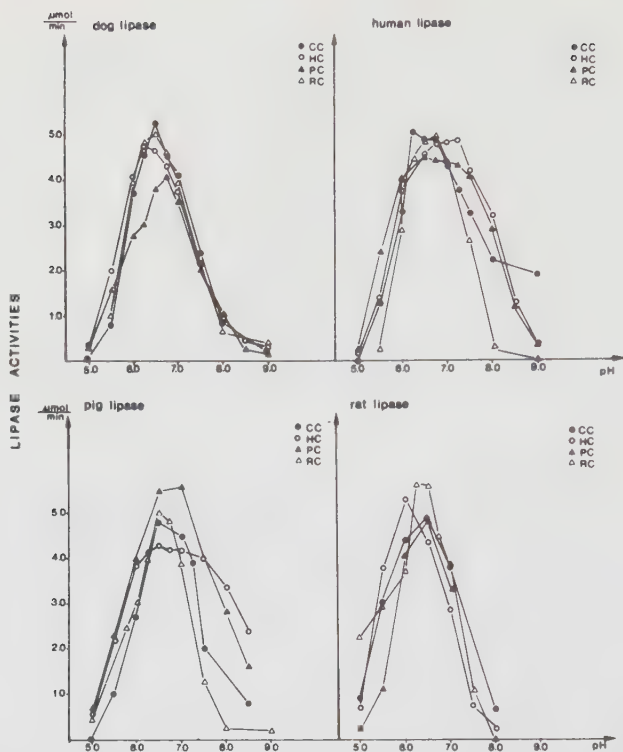


Fig. 1. Variation of lipase activity with pH. Purified human and porcine lipase and partially purified dog and rat lipases were assayed in the presence of canine colipase (CC); human colipase (HC); porcine colipase (PC) and rat colipase (RC). Each point is the mean of at least two measurements.

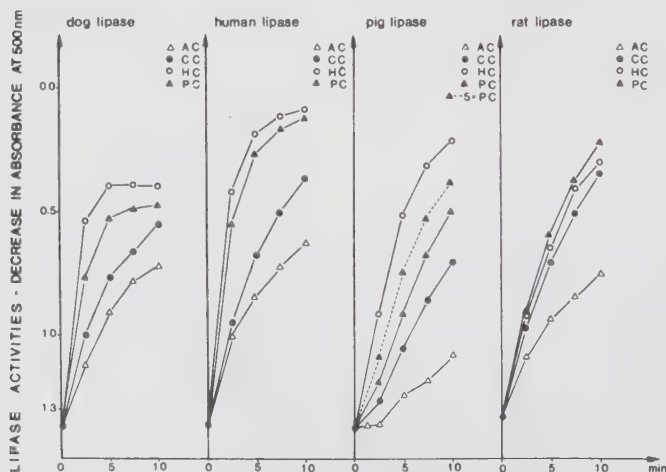


Fig. 2. Variation of lipase activity with incubation time. Purified human and porcine lipase, and partially purified dog and rat lipases were assayed in the absence of colipase (AC) and in the presence of canine colipase (CC), human colipase (HC) and porcine colipase (PC). Each point is the mean of at least two measurements.

The purified and partially purified colipases and lipases were used to determine any specific interaction between any one colipase and a lipase from the same or another species. The colipase and lipase solutions used for these studies were adjusted to contain approximately equal amounts of enzyme activity. The amount of colipase used was estimated to be about 10 pmol from its mol. wt (10,000 daltons) and the specific activity of colipase being approx 40,000 μ mol of butanoic acid released per min per mg protein.

Lipase, equivalent to about 20 pmol of lipase, giving about 10 μ mol free butanoic acid per minute at pH 8.0 in lipase buffer (see Methods section) was used.

RESULTS

The pH optima for different colipase and lipase combinations in 4 mM NaTDC are shown in Fig. 1 and summarized in Table 1. With a higher ratio of colipase to lipase broader pH optima were obtained. Colipases from dog, man, pig and rat activate lipases from all the species tested. The pH optima are in the range 6.0–7.25 and thus lower than those of the lipases alone, as previously found for porcine lipase (Borgström & Erlanson, 1973). There is no obvious correlation between the pH optimum and the pI for the different combinations (Table 2). To check if the method used to determine colipase and lipase activity can explain the specificity of canine colipase described by Lee (1978) a modification of the Vogel-Zieve method was employed (see Methods section). Fig. 2 shows that each lipase is also stimulated by any of the different colipases using this method. The decrease in optical density stopped after about 5 min with the absorbance equal to about 0.5 with canine lipase and human or porcine colipase. No inhibition could be obtained for human and/or rat lipase by canine colipase. The porcine lipase had low activity at this pH. Not even with 50 pmols of the homologous colipase could the same stimulation be obtained as with human colipase (see for comparison the activation of

human lipase by human and porcine colipase, Fig. 2). If a less-finely emulsified olive oil was used (e.g. $A_{500} \approx 0.3$), lipase stimulation by colipase was still obtained, but to a much smaller extent.

DISCUSSION

Colipase and lipase has been purified or partially purified from dog, man, pig and rat pancreatic gland or juice to investigate whether species' specificity exists. The results show that none of the colipases has any specificity for a certain lipase, except that for full activity a lipase possibly needs less of colipase from the same species than from others. Contrary to the results of Lee (1978) canine colipase was found to stimulate human and rat lipases. The findings in this study agree well with the results reported by others indicating no species' specificity. Bovine and porcine colipase activate bovine lipase to the same degree (Julien *et al.*, 1972), porcine colipase is an efficient cofactor for rat lipase (Borgström & Erlanson, 1973), pancreatic lipase from one species is activated by the addition of colipase from other species (Rathelot *et al.*, 1974; Vandermeers-Piret *et al.*, 1977), colipases from cow, horse and sheep stimulate ovine lipase (Canioni *et al.*, 1975), human lipase is also activated by other colipases than human (Caro *et al.*, 1977), shark colipase was found to activate porcine lipase (Patton *et al.*, 1978), and trout lipase is activated by porcine colipase (Leger *et al.*, 1979).

The method used by Lee to measure lipase activity is indirect and based on the clearing of an emulsion produced by the hydrolysis of the triglyceride emulsion. It is sensitive also to other factors, as to the particle size of the emulsion and pH which may effect the physical state of the assay system. Our results and those reported by the authors cited above are based on the titration of the fatty acids liberated by hydrolysis, and therefore are more specific. In the present paper we have also used the method of Vogel and Zieve, but have not been able to repeat the findings reported by Lee indicating that canine colipase does not restore activity of lipase from human and rat pancreas.

A definite explanation for the difference in results reported by Lee (1978) and those of the present investigation is not apparent. However, we wish to point out the following.

The experiments by Lee were undertaken at pH values at which the activity of the colipase-lipase system is low (see Fig. 1 of this paper) and the stimulation by colipase is rather unimportant due to a weak binding of colipase to lipase (Patton *et al.*, 1978). Furthermore, the concentration of canine colipase used by Lee was 10 times higher than that used in this study to activate human and rat lipase, and resulted in inhibition of lipase activity. This inhibition disappeared when the colipase concentration was reduced to a level which, however, did not stimulate the activity of bovine and canine lipases. These facts may throw some doubts on the purity of the canine colipase used by Lee.

We have now shown species independence, collaborating work described previously by several authors and have been unable to confirm a species specificity as suggested by Lee (1978).

Table 1. pH optimum of colipase-lipase in colipase buffer

	Colipase			
	Canine	Human	Porcine	Rat
Canine lipase	6.50	6.25	6.75	6.50
Human lipase	6.25	7.25	6.50	6.75
Porcine lipase	6.50	6.50	7.0	6.50
Rat lipase	6.50	6.0	6.50	6.25

Table 2. pI of different colipases and lipases

Canine	Colipase	7.9
	Lipase	5.8
Human	Colipase	I + II
	Lipase	I + II
Porcine	Colipase	I + II
	Lipase	I + II
Rat	Colipase	5.3†
	Lipase	I + II

* Values for procoplipases. Porcine colipases have a higher pI. Procolipase I has been used in these experiments.

† The pI of rat colipase was determined in 2% ampholine, pH 5–9, 1200 V, 4 mA and 48 hr, 110 ml column (L.K.B. Sweden).

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IMMUNOREACTIVE PANCREATIC COLIPASE, LIPASE AND PHOSPHOLIPASE A₂
IN HUMAN PLASMA AND URINE FROM HEALTHY INDIVIDUALS

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SUMMARY

A radioimmunoassay for each of the human pancreatic proteins (colipase, lipase and phospholipase A₂) is described. Determinations of the mean concentration of each protein in plasma and urine from healthy individuals were carried out with the radioimmunoassays. The values obtained in plasma were 0.5 nM (5.3 µg/l), 0.6 nM (32 µg/l) and 0.3 nM (4.3 µg/l) for colipase, lipase and phospholipase A₂, respectively. In urine the corresponding values were found to be 0.2 nM (2.4 µg/l), 0.09 nM (4.4 µg/l) and less than 0.017 nM (0.2 µg/l). No physical interaction between any of the three proteins and the lipid-particles of plasma was demonstrated by centrifugation experiments or gel filtration. Gel filtration of plasma, depleted of fat by centrifugation showed the proteins only in their monomeric form. The corresponding porcine proteins displayed a binding to anti-bodies against the human proteins, but with a lower affinity than the homologous interactions. The binding was weak but could differentiate between the porcine proforms and activated ones i.e. procolipase and colipase_{g7}.

INTRODUCTION

It has been known for some time that human plasma and urine contain enzymatic activity which is probably derived from the pancreatic gland (1), indicating a leakage of pancreatic proteins to the blood circulation. The concentrations of several of these proteins have been determined in human plasma and urine by various methods, mainly enzyme activity tests or radioimmunoassays. In the past, mostly trypsin (2,3) and pancreas-specific isoamylase (4) have been estimated, but recently the plasma-concentrations of some lipolytic pancreatic proteins have also been measured (5). The results from those studies indicate that there is normally a small release of pancreatic proteins into the circulation.

Acute pancreatitis, on the other hand, is generally associated with elevated levels of pancreatic proteins both in plasma and urine (6,7). Severe cell-damage is usually observed in patients with this condition. Both phospholipase A_2 (8) and lipase/colipase (9) have been proposed as agents responsible for these effects. Apart from acute pancreatitis, several other abdominal pathologies have been reported to raise the plasma levels of pancreatic proteins (10).

In this work we describe the development of simple radioimmunoassays for the determination of human pancreatic colipase, lipase and phospholipase A_2 . The concentrations of these proteins were determined in plasma and urine from healthy humans, and the size distribution of their antigenic activity in plasma was analysed.

MATERIALS AND METHODS

Proteins

The pancreatic proteins colipase₈₆, lipase and phospholipase A₂ were prepared from human pancreas as described (11,12,13). The proteins were found to be homogeneous according to the same criteria as presented previously. Bovine serum albumin (Cohn fraction V) was purchased from the United States Biochemical Corp., Cleveland, Ohio. The corresponding pure porcine proteins were gifts from Dr. A. Larsson and Dr. M. Lindström, Lund, Sweden.

Antibodies

Antisera against human colipase₈₆, lipase and phospholipase A₂ were raised in rabbits as described earlier (14). Purified antibodies were prepared by passing the antiserum through a column of Sepharose 4B, coupled to colipase₈₆, lipase or phospholipase A₂. The coupling of protein to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) was performed as suggested by Pharmacia (15). After washing the column the specific antibodies were eluted with 0.1M glycine-HCl pH 3.0. The eluted antibody solutions were concentrated using a PM-30 membrane (Amicon Corp., Lexington, Mass., USA) and centrifuged, after adjusting the pH to 7.4. Antibody solutions and whole antisera were kept frozen at -20°C until used.

Plasma and urine

Venous blood was collected in heparinized glass tubes. The blood specimens were drawn from 18 healthy, randomly chosen individuals, 10 females and 8 males, aged 25-40 years. There were no restrictions regarding eating before the samples were drawn. The blood was centrifuged and the clear plasma was stored frozen at -20°C until analyzed. Urine samples were obtained from the

same individuals, on one occasion for each person. Urine was also collected from one individual with renal tubular damage. The urine samples were also stored at -20°C until analyzed.

Labelling of proteins with ^{125}I

A modification of the chloramin-T method (16) was used for the labelling of proteins with ^{125}I . Typically, 0.2 nmoles of the protein were allowed to react with 0.5 mCi ^{125}I NaI (Amersham, Buckinghamshire, England), producing a radiolabelled protein with a specific activity of 0.2-0.3 mCi/nmole. After the reaction, free and protein-bound iodine were separated by dialysis of the mixture (bovine serum albumin added to 0.05%, w/v) against 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl and 0.02% NaN₃. The enzymatic activities of colipase and lipase were tested before and after labelling with ^{125}I . The exposure to chloramin-T and incorporation of iodine did not reduce the enzymatic activity of the two proteins. The low concentration of enzyme did not allow similar tests for phospholipase A₂.

Radioimmunoassay procedure

All radioimmunoassays were performed according to Plesner et al. (17), separating radiolabeled antigen bound to antibodies from non-bound antigen by precipitation with 10% polyethylene glycol-6000 (PEG). Dilutions of standards, antibodies, radiolabelled antigens and unknown samples were done with a 0.1 M phosphate-buffer, pH 7.5, also containing 0.1% (w/v) bovine serum albumin and 0.02% (w/v) NaN₃. 200 µl of standards or unknown samples were mixed with 100 µl of the radiolabelled antigen and 200 µl of the corresponding antibodies. After incubation overnight at room-temperature, 300 µl bovine serum and 1600 µl 15% (w/v) PEG, were added. The suspension was centrifuged at 1500xg for 30 min, after which the supernatant was discarded, and the radioactivity in the pellet was counted in a RIA gamma counter (LKB, Stockholm, Sweden). The standard inhibition curve was

plotted, with the radioactivity found in the pellet (expressed as the percentage of total radioactivity added to the sample) as ordinate and the logarithmic standard non-labelled antigen concentration as abscissa. All determinations were carried out at least in duplicate, and concentrations were calculated from plasma samples diluted four times, and urine samples diluted twice.

Centrifugation of human plasma

Human plasma was divided into a lipid-rich and a lipid-depleted fraction by centrifugation at 40,000xg and 4°C for 3 hours. The lipid-rich fraction (15% of the total volume) was harvested at the top after the centrifugation. The content of either endogeneous colipase, lipase and phospholipase A₂ or exogeneously added ¹²⁵I-colipase, ¹²⁵I-lipase and ¹²⁵I-phospholipase A₂ (incubation at room-temperature for 20 hours before centrifugation), was determined in the two fractions by radioimmunoassay or determination of radioactivity/ml.

Gel chromatography of human plasma

After centrifugation as described above, a portion of plasma (25 ml) was applied to a column (130 x 5 cm) packed with Sephadex G-200 superfine (Pharmacia Fine Chemicals, Uppsala, Sweden), and equilibrated with 20 mM Tris-HCl, pH 8, 0.15 M NaCl and 0.02% NaN₃. The column was eluted at 4°C with this buffer and fractions were collected. The total protein content of the fractions were estimated by U.V. absorbance at 280 nm. The fractions were pooled into 15 larger fractions, which were concentrated 30-50 times by ultrafiltration (18), and the concentrations of colipase, lipase and phospholipase A₂ were determined by radioimmunoassay. 0.7 ml aliquots of the centrifuged plasma were incubated for 20 hours at room-temperature with the ¹²⁵I labelled proteins separately (5 ng colipase, 20 ng lipase, 5 ng phospholipase A₂) and subsequently

subjected to gel chromatography on a Sephadex G-200 Superfine column (1.5 x 90 cm), equilibrated and eluted as above. Fractions were analysed for total protein and ^{125}I -activity.

RESULTS

Titration of antibodies

Figs. 1a,b,c show the results of the titration of antibodies against the three human antigens, respectively. Maximal binding capacity of colipase antibodies was about 85% of ^{125}I -colipase₈₆ and the dilution 1:100,000 of anti-colipase antibodies was chosen. The maximal binding capacity of lipase antibodies was 90% of ^{125}I -lipase and the dilution 1:8000 was used. Phospholipase A₂ antibodies were maximally bound 70% of the ^{125}I -phospholipase A₂ and antiphospholipase A₂ antibodies were diluted 1:20,000.

Radioimmunoassay

Figs. 2a,b,c show the standard curves for human colipase₈₆, lipase and phospholipase A₂. Detection limit (defined as the smallest quantity of each antigen that can be distinguished from zero) was determined to be 0.02 nmol/l (0.2 µg/l) colipase, 0.004 nmol/l (0.2 µg/l) lipase and 0.015 nmol/l (0.2 µg/l) phospholipase A₂, when the antibody dilutions stated in the previous section were used. The concentration range of standards for all three antigens was 0.1 µg/l to 200 µg/l.

Colipase, lipase and phospholipase A₂ in plasma and urine

Table 1 shows the values obtained for the mean concentrations of each antigen and the concentration in the corresponding pooled samples. As control, the capacity of rabbit serum to bind each of the antigens was examined, and found not to react in the radioimmunoassays. Figure 3 compares the colipase in urine from a patient with renal tubular damage with standard colipase₈₆. The curves are parallel.

The size-distribution of the antigenic activity of the three proteins was studied by gel chromatography of plasma. Figure 4 shows the specific concentrations of colipase, lipase and phospholipase A₂ in the concentrated fractions. Apparently, no activity other than the monomers was revealed by the radioimmunoassays for any of the three proteins.

Lipid-rich and lipid-depleted plasma were prepared by high-speed centrifugation. No differences were seen between the concentrations of colipase, lipase and phospholipase A₂ in the two fractions, as determined by radioimmunoassay. All three proteins, labelled with ¹²⁵I, distributed in equal concentrations in the lipid-rich and lipid-depleted fractions of plasma, when added exogeneously, one at a time. The size-distribution of ¹²⁵I labelled colipase₈₆, lipase or phospholipase A₂, incubated with non-centrifuged plasma, was studied by gel-chromatography. All three proteins were eluted at a position corresponding to the monomers. No other form was noted for colipase and only minor quantities of the radioactivity of lipase and phospholipase A₂ appeared in the void volume.

Cross reactivity with the porcine proteins

100 µl ¹²⁵I labelled human antigen, 200 µl anti-human protein-antibodies and 200 µl of the corresponding porcine protein (conc. range 1 µg/1-2 mg/1) were incubated as described for each of the homologous systems. Porcine colipase₈₇, lipase and phospholipase A₂ bound approximately 30% of the human antibodies at a concentration corresponding to the human proteins.

DISCUSSION

A fluid-phase radioimmunoassay, involving relatively few and simple steps, has been applied to the estimation of colipase, lipase and phospholipase A₂ concentration in human plasma and urine. The radioimmunoassay, as described by Plesner et al. (17), includes molecular exclusion of antibody-bound antigens from the solution by addition of polyethylene glycol. This method is applicable to proteins with a relatively small size (less than 50,000-100,000 daltons), and can thus be used for these three molecules *appr.* (M_w : 10,000, 50,000, 14,000, respectively). The three different radioimmunoassays were specific with regard to each other. The radioimmunoassays were not affected by rabbit sera, suggesting a lack of non-specific interactions by plasma-proteins. Also the antigenic activity in plasma was apparently restricted to only one component (Fig. 4), indicating monospecificity of the radioimmunoassays. These components were able to bind a substantial fraction of the antibodies, which clearly suggests that the components represent colipase, lipase and phospholipase A₂, and not any minor components in the original preparations of the proteins to which the antisera were raised.

The concentration of colipase in normal human plasma was estimated to be 5.27 $\mu\text{g/l}$ (S.D.= 2.8 $\mu\text{g/l}$), and in normal human urine 2.38 $\mu\text{g/l}$ (S.D.= 1.9 $\mu\text{g/l}$). This is lower than the detection limit (6.5 $\mu\text{g/l}$) of Junge (20), who did not find any colipase in normal human serum or urine with a turbidimetric method. We found 32.2 $\mu\text{g/l}$ (S.D.= 28 $\mu\text{g/l}$) and 4.41 $\mu\text{g/l}$ (S.D.= 5.0 $\mu\text{g/l}$) of pancreatic lipase in human plasma and urine, respectively. These results correlate well with those obtained by Grenner et al. (21), who used a solid phase enzyme immunoassay (7-37 $\mu\text{g/l}$). They did not report any lipase in urine of humans. The amount of human phospholipase A₂ was found to be 4.25 $\mu\text{g/l}$ (S.D.= 1.3 $\mu\text{g/l}$) in plasma. Hardly any urinary phospholipase A₂ was detected

(detection limit was approximately 0.2 $\mu\text{g/l}$). Investigators in Japan (8) have reported similar plasma-concentrations of this protein, 2.0-7.9 $\mu\text{g/l}$, average concentration 5.1 $\mu\text{g/l}$ (S.D.=1.7 $\mu\text{g/l}$), using a radioimmunoassay. No determinations of urinary phospholipase A₂ were reported.

Leger et al. (19) found that the colipase of pig plasma was not monomeric. The support for this observation, however, is weak because with gel filtration of one ml pig plasma (colipase conc. $\approx$ 28 ng/ml, i.e. about 5 times that in human) they found the colipase in only one fraction (1.3 ml, containing 1 ng/ml). Human plasma contains only monomeric colipase, lipase and phospholipase A₂ (Fig. 4). No aggregations or complexes with other molecules were suggested by the elution volumes on gel chromatography. The concentrations of the three proteins did not differ in plasma depleted or enriched in its lipid content by centrifugation, suggesting that colipase, lipase and phospholipase A₂ do not associate with lipid particles of very low density. This was also confirmed using exogeneously added ¹²⁵I labelled proteins.

The three lipolytic proteins investigated here, are small enough to be filtered through the renal glomeruli, and reabsorbed by the renal tubules (22). In fact, the plasma level of lipase is increased in chronic primary renal failure (23) and the amount of colipase in urine is elevated in patients with renal tubular damage, above the level found in normal plasma. The similarity between the inhibition curves obtained by standard colipase₈₆ and this urine, suggest that the colipase found in this urine was not degraded in plasma and/or kidney. The reabsorption rate by the renal tubules is determined partly by the net charge of the particular protein, positively charged proteins being more readily reabsorbed than negatively charged proteins. Thus a large fraction of phospholipase A₂, positively charged at physiological pH, would be expected to be reabsorbed from the glomerular fil-

trate yielding low urinary concentrations in the final urine, while the opposite would be expected for colipase and lipase. This is in agreement with the results obtained in this work. Similar results have been shown for trypsinogen and trypsin; canine cationic trypsinogen and trypsin are not detected in urine but the anionic forms are (24). However, it must be emphasized that the data presented here are based on only a few measurements and no attempts were made to investigate further the metabolism and kinetics of the renal handling of the three proteins.

Porcine colipase, lipase and phospholipase A₂ were able to interact immunologically with the corresponding human proteins. The affinity, however, was lower than that of the homologous antigen-antibody complexes. This cross-reactivity confirms the structural homology between these proteins (13,25,26).

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Table 1

Concentrations of colipase, lipase and phospholipase A₂ in human plasma and urine.

Antigen	Plasma ($\mu\text{g/l}$)		Urine ($\mu\text{g/l}$)	
	Pooled samples ¹⁾	Mean of separate determinations (S.D.) ²⁾	Pooled samples ¹⁾	Mean of separate determinations (S.D.) ²⁾
Colipase	4.27	5.27 (2.8)	1.87	2.38 (1.9)
Lipase	35.2	32.2 (28)	<1	4.41 (5.0)
Phospholipase A ₂	3.66	4.25 (1.3)	<0.2	0.24 (0.1)

¹⁾Aliquots from 18 different donors were pooled before the determinations.

²⁾Aliquots from 18 different donors were determined separately.

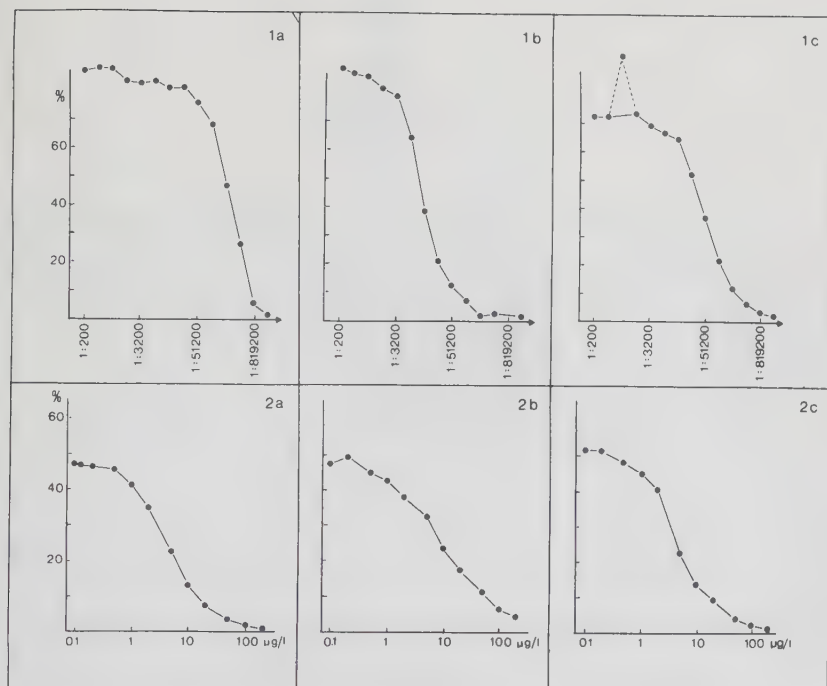


Fig. 1. % Antibody-bound/total radioactivity when a constant amount of ^{125}I -labelled colipase₈₆ (a), lipase (b) and phospholipase A₂ (c) is incubated with increasing dilutions of antibodies.

Fig. 2. Standard calibration curves for radioimmunoassays of each of colipase₈₆ (a), lipase (b) and phospholipase A₂ (c). % Antibody-bound/total radioactivity versus increasing concentrations of non-labelled antigen.

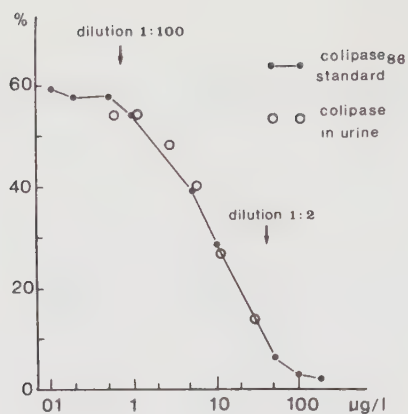


Fig. 3. Displacement of radiolabelled colipase₈₆ bound to anti-colipase antibodies, by standard non-labelled colipase₈₆ and urine from a patient with renal tubular damage at different dilutions.

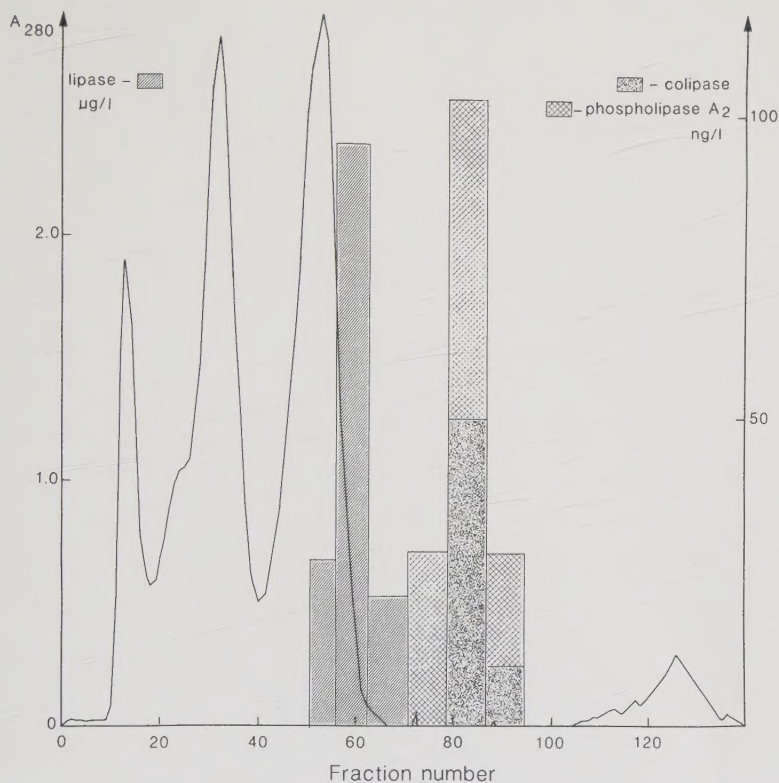
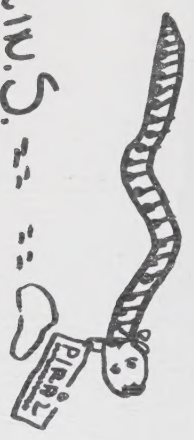
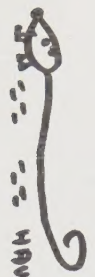
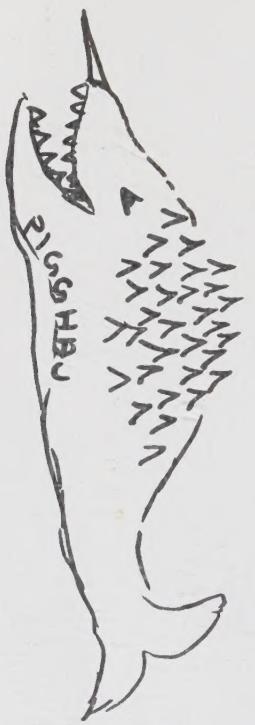


Fig. 4. Gel filtration of 25 ml centrifuged human normal plasma on a Sephadex G-200 column. Fractions were pooled and concentrated and the amounts of colipase, lipase and phospholipase A₂ were determined by radioimmunoassay.



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